THE JOURNAL OF PHYSIOLOGY

EDITED FOR

THE PHYSIOLOGICAL SOCIETY

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VOL. 93

1938

LONDON
CAMBRIDGE UNIVERSITY PRESS

74 18
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PRINTED IN GREAT BRITAIN

9599

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J. Physiol. (1938) 93, 1–9 612.332.73:612.112.11

THE ROLE OF LEUCOCYTES IN FAT ABSORPTION

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(*Received* 24 *January* 1938)

In 1867 Arnstein noted the presence of fat in leucocytes of the intestinal epithelium. Although the amount of fat in them increased after fat feeding, he did not attribute to them any important role in fat absorption. In fresh preparations he observed migration of these cells from the epithelium to the lumen of the intestine. Zawarykin [1883] and Schafer [1885] came to the conclusion that leucocytes transport fat from the lumen to the lacteals. In a careful study of the intestinal epithelium, R. Heidenhain [1888] described a greater diversity of types of leucocytes. He showed that some contained true fat droplets, while others contained inclusions which blackened with osmium tetroxide, were insoluble in ether and stained with acid fuchsin. Both he and Grünhagen [1887] denied the fat transport theory. Schafer [1912] admitted that some of the droplets, which he had regarded as fatty in nature, were not true fat. He did not describe the distribution of the latter. Fat transport was now stated to take place from the epithelial cells, not from the lumen of the intestine, by means of lymph corpuscles. He still referred to lymph corpuscles and leucocytes synonymously, although the various types of leucocytes both of the blood and intestinal epithelium had already been accurately described. He regarded the fat transport theory as applicable to mammals, in which the most frequently occurring type of leucocyte of the intestinal epithelium is the lymphocyte. Consequently it is the latter which is usually referred to in connexion with the fat transport theory.

Although Zawarykin [1883] and R. Heidenhain [1888] made some differentiation of leucocyte types, Hardy & Wesbrook [1895] first made an accurate study of them. In the drawings and description of microscopical preparations by Thanhoffer [1874] and nearly all subsequent workers in this field (including Schafer) it is often possible to recognize the various types of leucocytes of the intestinal epithelium. Mottram *et al.* [1922] subjected rats to radium emanations and found that in such

animals, which presumably suffered from lymphopenia, no fat absorption occurred. For reasons unstated, they drew the conclusion that, although lymphocytes play a role in fat absorption, it is not that of fat transport.

The object of the present work was to determine whether any or all of the types of leucocyte of the intestinal epithelium which have now been recognized take part in fat absorption. Fat absorption in normal frogs has therefore been studied and in addition the effect of benzene—which causes leucopenia—on fat absorption in the rabbit has been examined.

Methods

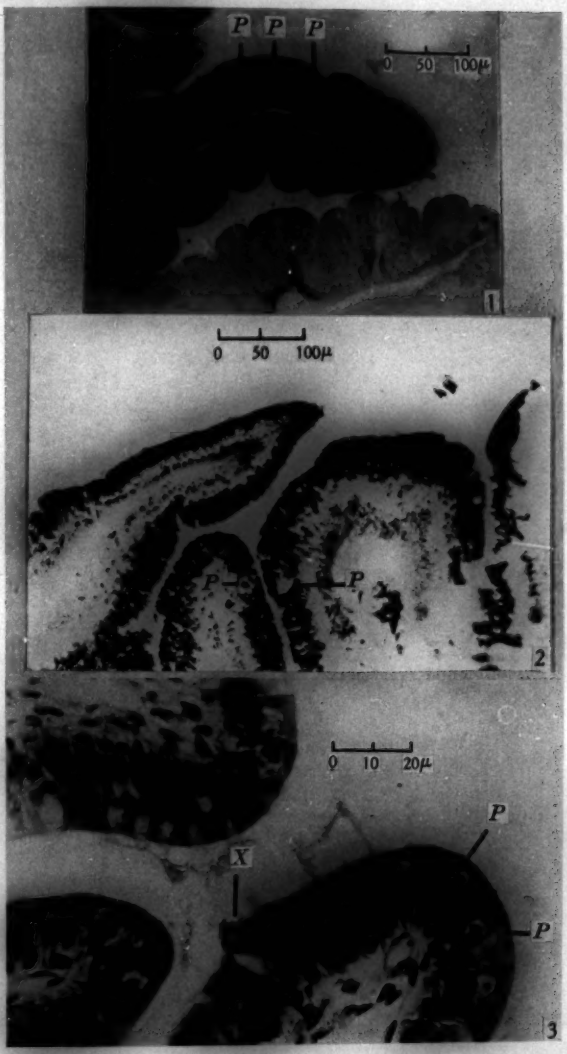
Spring and summer frogs weighing 10–15 g. were used. Some of these had been kept at a temperature of 4° C. for periods up to 28 days. Olive oil (about 0·1 c.c.) was dropped into the mouth, and the animals were killed at intervals after feeding. Portions of various segments of the intestine were fixed in Heidenhain's "Susa" [Heidenhain, 1916] and in absolute alcohol. Sections of the Susa-fixed material were stained by hæmatoxylin-eosin and Masson's iron hæmatoxylin-ponceau 2*R*-acid fuchsin-light green [Masson, 1923]. Alcohol-fixed material was stained by methyl green-pyronin. Other material was fixed in 1 p.c. osmium tetroxide or 1 p.c. osmium tetroxide and 1 p.c. chromic acid for 7 days, washed for 7 days, then embedded in gelatine, hardened in formaldehyde, and cut frozen. Some sections were counterstained in Scharlach R.

Rabbits were given a daily subcutaneous injection of 1–10 c.c. of "benzole cryst." (of Harrington) until the blood leucocyte count was reduced to about 1000–2000 c.mm. Then 5–15 c.c. of olive oil were fed by stomach tube to normal and benzene-poisoned animals. The fæces were collected daily before and after the fat feeding. Each 24 hr. portion was separated by hand, acidified with hydrochloric acid, dried at 110°, powdered and weighed. A sample of each was continuously extracted with light petroleum in a Soxhlet apparatus for 3 hr. The percentage of petroleum-soluble substance associated with the non-fat residue was calculated for the 2 days before, and for several days after, the fat feeding. Hence by subtracting the total amount of petroleum-soluble substance that would have been associated with the non-fatty residue of the fæces after fat feeding from the total fat of the fæces, the total of the unabsorbed olive oil was obtained. The greater part appeared in the fæces between 24 and 48 hr. after the feeding. After 2–5 days, when little excess fat was present in the fæces, olive oil was again fed and the animals killed 7 hr. later by a blow on the head. A complete post-mortem examination was made. Small pieces from the middle of the small intestine were cut open and fixed in 5 p.c. formaldehyde in 0·9 p.c. sodium chloride solution for 24 hr., and then embedded in gelatine. Sections were cut at 15 μ., stained in Sudan III and mounted in Farrant's medium. Control material was fixed in "Susa" and embedded in paraffin. Sections were stained by routine methods.

Results

Fat absorption in frogs

The epithelial cells became elongated during fat absorption owing to the large amount of fat that they contained (Pl. I, figs. 1, 2). The maximum deposit was usually at the 12th hour after feeding. The size of



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the droplets was much smaller in the animals which had been kept at a low temperature. The presence of a few fat droplets in the epithelial cells even at the end of a fast of at least 28 days supports the view of Rony *et al.* [1933] that a continuous excretion and reabsorption of fat occurs in the intestine.

From sections stained by routine methods it is possible to distinguish three types of leucocytes in the intestinal epithelium.

(1) *Lymphocytes.* These are present between the epithelial cells and in the subepithelial connective tissue. The number found during the various stages of fat absorption did not differ from that present during starvation.

(2) *Eosinophils.* Although occurring in the epithelium, especially near the basal border, these are more numerous in the subepithelial connective tissue. They decrease in number during fat absorption and can easily be recognized by their closely packed refringent granules.

(3) *Phagocytic cells.* These are large (diameter 5–20μ.). The nucleus is flattened or crescentic and peripherally placed. One or more large vacuoles are present in the cytoplasm. Various cell inclusions are visible, some of which appear to be red or white blood corpuscles in various stages of digestion. The ferrocyanide reaction reveals the occasional presence of iron-containing granules. The cells found near the base of the epithelium are small, those near the striated border larger. Some cells can be seen migrating through the striated border (Pl. I, fig. 3) and other large ones within the lumen of the intestine. During fat absorption, a decrease occurs in the number of these phagocytic cells. After vital staining with trypan blue, the dye was identified in these cells, indicating that they may be members of the reticulo-endothelial system.

Sections of material fixed with osmium tetroxide-chromic acid showed no black or brown granules in the lymphocytes. The oxyphil granules of the eosinophils stain light brown. Neither the number nor the intensity of staining of these granules varies during fat absorption. Within the phagocytic cells can be seen black droplets of varying size and also large brown inclusions corresponding to the R.B.C. fragments. The black droplets are present even after the animal has starved at least 28 days. The number and size of these droplets present within a phagocytic cell increased during fat absorption although the total number of these cells decreased (Pl. I, figs. 1, 2). Very rarely, cells containing black droplets can be found in the subepithelial connective tissue or lining the walls of the lacteals. They are much smaller than the phagocytic cells of the epithelium.

Sections of material fixed in osmium tetroxide alone show a slightly different appearance. A heavy, general, brownish-black staining obscures much detail. The oxyphil granules of the eosinophils are dark brown and, if the lighting is far removed from critical illumination, they appear black.

After treatment with ether for 12 hr., the black droplets within the phagocytic cells disappear but the dark brown granules of the eosinophils are unaffected.

In some control material, fixed in formaldehyde and stained in Scharlach R, red staining droplets can be seen in the epithelial cells and in the phagocytic cells. The oxyphil granules of the eosinophils stain light yellow.

Effect of benzene on fat absorption

The benzene-injected animals became very weak. "90 p.c. benzole" and chemically pure benzene were found to be as toxic as "benzole cryst." and no more potent in leucotoxic activity. Thiophene and dioxane are both chemically related to benzene and the former is a constant impurity in "benzole". Neither was found to act as a leucotoxin when administered in non-fatal doses.

TABLE I

Serial no.	W.B.C.'s/c.mm. blood	Olive oil fed c.c.	Olive oil absorbed, p.c.	Benzene injected, c.c.
F.A. 3	8900	15	76	0
F.A. 5	7700	5	70	0
F.A. 2	400	15	54	15
F.A. 1	2500	15	48	33
F.A. 7	1150	5	40	44

In differential W.B.C. counts of other benzene-poisoned rabbits, it was found that the reduction in the lymphocyte count was roughly proportional to the reduction in the total W.B.C. count.

The lymphoid tissue of lymph glands and appendix showed very marked depopulation. Few normal, mature lymphocytes were visible either here or in the blood. From sections of other organs, the results of Selling [1916] were confirmed. In sections of the intestine of the normal fat-fed animal F.A. 5, much fat was visible within the epithelial cells and in the lacteals but none within the lymphocytes of the epithelium. In the benzene-poisoned animal F.A. 7 there was much less fat present in the epithelial cells and slightly less in the lacteals.

In the control sections, it was found that the number of lymphocytes in the intestinal epithelium had not been markedly diminished by the benzene poisoning. However, the nuclei of the lymphocytes both here and in the blood had become definitely pycnotic.

Discussion

It does not appear likely, from the experiments with frogs, that lymphocytes are involved in fat transport, as no significant change has been noted in the relative number present during the various conditions, and no fat could be identified in them by the methods used. Winiwater [1930] found that prolonged feeding on exclusively fatty diet does not increase the number of lymphocytes in the intestinal epithelium of mice. Goldmann [1933], using special methods, was unable to find fat in lymphocytes although he identified it in all the other types of leucocytes. Again, in the experiments of Clark & Clark [1917], the cells which took up the fat droplets were never identified as lymphocytes or even as leucocytes. Although Schafer attributed fat transport activities to lymph corpuscles, it does not appear certain from his drawings that true lymphocytes were engaged in this activity at all.

The results of the experiments on rabbits, which at first sight support the fat transport theory of lymphocytic activity, in reality tend to disprove it. During fat absorption, neutral fat is laid down within the epithelial cells whenever the rate of entry of fat into these cells is greater than the rate of removal from them [Noll, 1910]. In the benzene-poisoned animals the rate of entry of fat must be diminished because, after administration of fat, more fat appears in the fæces of these subjects than in the fæces of normal animals. The greatest decrease in this fat-absorbing capability was 30 p.c. in animal F.A. 7. If the rate of removal of fat from the epithelial cells had been interfered with to a greater extent than this, more fat would have been laid down within these cells than in a normal animal. As the amount of such fat was found to be less than normal, the interference with the rate of removal must have been less than 30 p.c. If the lymphocytes of the intestinal epithelium play any part in fat transport, it would have been expected that the reduction in the rate of removal of fat would have been large owing to the pycnotic change in their nuclei and the small reserves of lymphocytes in blood and lymphoid tissue.

Schafer [1885] has also suggested that the lymphocytes carry the fat direct from the lumen of the intestine to the lacteals where they disintegrate. The experiments described above would confirm this theory, were it not that no fat was visible in the lymphocytes of the epithelium.

In the benzene-poisoned animal F.A. 7, 2 g. of fat were absorbed. If this amount is transported by lymphocytes which disintegrate after

migration to the lacteals, so large a destruction should have led to a rapid depletion of the lymphocytes of the intestinal epithelium; for in such an animal the reserve of lymphocytes in the blood and lymphatic organs was much decreased. No such depletion was, however, observed.

Mottram *et al.* [1922] considered that their experiments indicated that lymphocytes play a role in fat absorption other than that of fat transport. Presumably they were convinced that failure of fat absorption in their rats was due to the depletion of lymphocytes in the lymphoid organs and not to a more general effect of the radium emanations. A similar conclusion cannot be drawn from the experiments described here. Although only three benzene-poisoned rabbits were used, it appears from Table I that the failure of fat absorption bears a nearer relationship to the total amount of benzene injected than it does to the lymphopenia induced. So the failure of fat absorption is probably due to a more general toxic effect of benzene poisoning and no role need be attributed to lymphocytes in fat absorption.

The second type of leucocyte to be considered is the eosinophil. These occur in a much higher proportion in the blood of the frog than in that of mammals. The granules of these cells do not stain with Scharlach R and so are probably not true lipide in nature. In preparations fixed in osmium tetroxide together with chromic acid, the granules are yellow, but with osmium tetroxide alone they are brown, and if the lighting does not approximate to critical illumination they appear to be black. It is interesting to note that both Zawarykin and Schafer used osmium tetroxide alone and apparently from the drawings and description regarded these granules as absorbed fat. Grünhagen [1887] employed Flemming's solution and did not regard these granules as fat. Heidenhain [1888], using osmium tetroxide only, described black granules in such cells but did not regard them as fatty in nature as they were insoluble in ether and stained with acid fuchsin. It appears that Schafer [1885] was referring to these eosinophils when he stated that the fat-containing cells became smaller as they migrated from the surface through the epithelium. His larger forms near the surface would then correspond to what are here described as phagocytic cells. Schafer [1912] admitted that some of the osmium-stained granules of leucocytes were, as Heidenhain stated, insoluble in ether and possibly not of true lipide nature. From the experiments here described, it appears that such ether-insoluble granules occur mainly in the eosinophils and the ether-soluble ones only in the phagocytic cells. Hence it is unlikely that eosinophil cells are concerned in fat transport.

The phagocytic cells of the epithelium were first described by Arnstein [1867]. He noted that they contained fat droplets and observed them migrating in fresh preparations from the epithelium into the lumen of the intestine. R. Heidenhain [1888] described degenerating red and white blood corpuscles within them. Hardy & Wesbrook [1895] pointed out that contained disintegrating eosinophils may give them the appearance of modified eosinophils. Bacteria [Ruffer, 1890], iron [Macallum, 1894], methylene blue [Champy, 1911] and vital dyes [v. Möllendorf, 1925] have all been noted within them. There is little accord as to the direction of migration, and such cells may well be concerned in either absorption or secretion. The result of the experiments here recorded are best interpreted as indicating that the phagocytic cells migrate through the epithelium, becoming larger as they approach the striated border; they take up fat in addition to other particulate matter and discharge it when they disintegrate within the lumen of the intestine. But the only direct evidence of migration is that of Arnstein [1867] who actually saw them entering the lumen. There is no evidence for the assumption that they are the precursors of any type of subepithelial cell, lymphocyte or eosinophil, which Schafer both in his description and drawings implied. The nature of these cells is discussed fully by Patzelt [1936].

The suggestion offered to account for the occurrence of fat in these cells is that they take up fat, just as v. Möllendorf [1925] considered that they take up vital dyes, owing to their phagocytic activity. This phagocytosis and migration into the lumen would involve a very minor inefficiency in fat absorption. It must be remembered that, in addition to fat, vital dyes, iron, disintegrating blood corpuscles and possibly other products of excretion will be removed coincidently.

Fat was occasionally seen in subepithelial cells, probably histiocytic and definitely not lymphoid in nature. Zawarykin [1883] described similar cells containing large fat droplets even in Meissner's plexus. Farkas & Thanhoffer [1933], using Flemming's fixative, described a similar distribution. Weiner [1932] also noted such cells. Macallum [1894] found iron and fat, and Wassiljeff [1925] trypan blue in them. Onozaki [1936] has found them recently in the colon of the dog. Actually such fat-containing cells may be seen not only in the connective tissue of small and large intestine but also in that of skin, stomach and submaxillary gland. It is unlikely that these histiocytic cells, relatively small in number, could play any large part in fat absorption. It is not unlikely that they are precursors of the phagocytic cells which have also been shown to take up vital dyes.

Summary

1. No fat can be detected in lymphocytes of the intestinal epithelium of the frog.

2. The oxyphil granules of the eosinophil leucocytes may easily be mistaken for fat droplets.

3. The phagocytic cells are the only leucocytes of the epithelium which contain fat. This fat is carried back into the lumen by migration of these cells.

4. Subepithelial cells, probably histiocytic in nature, may contain fat.

5. Benzene poisoning decreases the fat-absorbing capability of rabbits and decreases the number of lymphocytes in the blood stream and lymphatic organs.

6. Because no fat could be identified within lymphocytes and because in the benzene-poisoned animals there was no accumulation of fat within the epithelial cells or depletion of lymphocytes of the mucosa, it is concluded that lymphocytes do not transport fat from the epithelial cells or from the lumen of the intestine to the lacteals.

7. The failure of fat absorption appears to be due to a general toxic effect of benzene poisoning.

8. There is no evidence that any type of leucocyte plays an important part in the transference of fat from the epithelium to the lacteals.

My thanks are due to Dr H. M. Carleton for having suggested this problem and for much help and criticism, also to Prof. Sir Charles Sherrington, Prof. R. A. Peters and the late Prof. Dreyer for permission to work in their respective departments, and to Dr R. B. Fisher for help with fæcal extractions.

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EXPLANATION OF PLATE I

Fig. 1. Lower part of intestine of normal frog 12 hr. after fat feeding. Fixation 1 p.c. osmium tetroxide–1 p.c. chromic acid. Three phagocytic cells (*P*) containing fat clearly distinguishable.

Fig. 2. Upper part of intestine of normal frog 24 hr. after fat feeding. Same fixation. Epithelial cells now heavily loaded with fat. Two phagocytic cells (*P*) distinguishable, one of which contains fat droplets.

Fig. 3. Lower part of intestine of frog, which had previously been kept at a low temperature, 5 hr. after fat feeding. One phagocytic cell (*X*) leaving epithelium. Another (*P*) and other unmarked ones containing various inclusions.

J. Physiol. (1938) 93, 10–23 612.282

ON THE MECHANISM OF PRODUCTION, AND THE PHYSIOLOGICAL SIGNIFICANCE OF "APNEUSIS"

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(*Received* 8 *February* 1938)

According to Markwald [1887, 1890], when bilateral division of the vagi in a rabbit is preceded or followed by transection of the brain stem immediately behind the posterior colliculi, the respiration is profoundly altered, it ceases to be rhythmical, and consists of an irregular series of long powerful inspiratory "cramps", each of several seconds' or minutes' duration and only interrupted at irregular intervals by short expiratory pauses. If the nucleus of the trigeminus nerve is intact and functional, the inspiratory cramps begin gradually to become shorter, eventually being reduced to powerful, cramp-like respiratory acts of a few seconds' duration, following one after the other in a more or less regular, rhythmical series. If, however, the nucleus of the trigeminus nerve has been damaged, as shown by the disappearance of the corneal and nose reflexes, the inspiratory cramps remain long and irregular and lead to death from asphyxia. When division of the vagi, on the other hand, is performed in an animal decerebrated just in front of the posterior colliculi, or at any higher level, the effects on respiration are not different from those which are usually observed after vagotomy in an animal with the central nervous system intact: i.e. they simply consist in a deepening and slowing of the breathing. Markwald concluded that in the posterior colliculi there is a centre inhibitory to inspiration, whose action becomes clear when the inhibitory activity of the vagi is abolished by their section. He further suggested that the nucleus of the trigeminus nerve, when both the vagi and the posterior colliculi had been put out of action, would acquire a new tonus and exercise a rhythmical inhibitory influence, though a less efficient one, upon the inspiratory centre.

Markwald's findings were confirmed by Loewy [1888], Asher & Lüscher [1899], Langendorff [1888] and others. Lewandowsky [1896], while confirming the inhibitory influence on inspiration of the posterior colliculi, could not agree that the nucleus of the trigeminus nerve played any part in reducing the duration of the inspiratory cramps, since this modification of the cramps was not constant and in any case it occurred after sections in the lowest parts of the pons.

More recently, after an extensive study in the cat Lumsden [1923*a*, 1923*b*] arrived at results which differ in some important points from those described above. First, he found that section of the brain stem immediately behind the posterior colliculi did not modify the usual effects of division of the vagi, and saw therefore no necessity for postulating in the posterior colliculi the existence of a centre inhibitory to inspiration. According to Lumsden the inspiratory cramps described by Markwald appeared only when the section fell some millimetres below the upper limit of the pons, whether the vagi were intact or cut. When the brain stem was cut below the striæ acousticæ, inspiratory cramps ceased, and respiration consisted of a more or less regular series of gasps, i.e. of quick inspirations, beginning and ending abruptly and followed by passive expiration. Lumsden concluded that the centre causing the latter type of respiration represented a sort of primitive centre, the "gasping" centre which had been superseded in the course of evolution by two higher centres, (i) an inspiratory centre at the level of the striæ acousticæ, the "apneustic" centre, on whose unrestrained activity the long inspiratory cramps, or "apneuses" depended, and (ii) a centre in the upper region of the pons rhythmically inhibiting the activity of the "apneustic" centre and thus producing normal respiration or pneumotaxy. This he called the "pneumotaxic" centre.

Henderson & Sweet [1929] and Teregulow [1929] repeated the investigation in the cat and obtained results different from those described by Lumsden, but similar to those observed by Markwald in the rabbit, i.e. they found (i) that the inspiratory cramps, or "apneuses", appeared only after section of the vagi, (ii) that it was not necessary to cut the brain stem as low as some millimetres below the upper limit of the pons, as found by Lumsden, a section just behind the posterior colliculi being sufficient. Henderson & Sweet, however, differ from all previous investigators in the interpretation of apneusis, which they regard not as an exaggeration of the normal inspiratory activity, but as a manifestation of decerebrate rigidity, extended to the inspiratory muscles, and having no relation with respiratory activities proper. To

prove their contention, they cut the rubrospinal tracts in a vagotomized cat by a median longitudinal incision of the mid-brain reaching down to the upper part of the pons, when full apneuses immediately developed. To account for the fact that when the vagi are intact apneuses are not seen, they admitted that these nerves have a specific inhibitory influence upon those foci of decerebrate rigidity which control the inspiratory muscles. Hess [1931] also takes the same view of apneusis as Henderson & Sweet, i.e. he regards it as a phenomenon of decerebrate rigidity.

A third hypothesis on apneusis was put forward by Barcroft [1934], who regarded the gasp as the fundamental phenomenon of respiration and apneusis as a modified, interrupted gasp. Barcroft suggested that under normal conditions the gasp is damped or smothered into normal respiration, and that this damping is brought about by afferent inhibitory impulses set up by the very act of inspiration, in the whole respiratory system, including the respiratory muscles. The reflex arcs concerned reach the brain stem at different levels, probably up to the level of the posterior colliculi, and the meaning of "apneusis" is that the afferent inhibitory influences still reaching the "gasping" centre after division of the vagi and section in the pons are not sufficiently powerful to break the gasp, but only to interrupt it half-way.

From the above account it is seen that although the phenomenon of "apneusis" has often engaged the attention of physiologists, there still prevails a great degree of uncertainty as to the mechanism of its production and as to its physiological significance. The object of the present research was to obtain more accurate information about the experimental conditions under which apneusis ensues, and to subject to experiment the various hypotheses put forward.

Experimental

The research was made on cats to the number of forty-six. At the beginning of each experiment the animals were decerebrated by the trephine method, under ether anæsthesia, by intercollicular transection of the mid-brain passing, ventrally, 2 or 3 mm. in front of the pons. The tentorium cerebelli was then removed. For all subsequent sections in the brain stem very sharp cutting instruments were used, and, when necessary, the animal was temporarily placed again under slight ether anæsthesia. Intracranial division of the cerebral nerves was effected by very gradual compression by forceps until they were completely crushed. This method had advantages over acute section, in that it did not seem to disturb the animal, and that it caused very little bleeding.

The same procedure, i.e. crushing by slow gradual compression, was employed for dividing the spinal cord, thus dispensing with the necessity, otherwise, of putting the animal again under anæsthesia during the operation. The respiration was recorded by means of tambours.

The site of the centre which inhibits apneusis

Section of the brain stem immediately behind the posterior colliculi. Section at this level, passing ventrally through the superior border of the pons, caused no appreciable alteration of quiet breathing. The present experiments, therefore, do not support the findings of Markwald [1890], Lewandowsky [1896], Pike & Coombs [1918], nor those of Trevan & Boock [1922], according to whom the operation leads to a deepening and slowing of respiration similar to that observed after vagotomy.

After subsequent bilateral vagotomy, respiration became deeper and slower, but continued regular, and the effect was in no way different from that observed after simple vagotomy with the central nervous system intact. This was particularly well seen in those experiments in which the vagi had been divided before making the brain section. It was then seen that, while, in the limbs, neck and back a full degree of decerebrate rigidity developed, the chest, on the contrary, did not undergo any change and remained quiescent in the expiratory position, while respiration continued to be purely diaphragmatic, as before. Also the rate and depth of breathing remained absolutely unmodified.

With the exception of Lumsden, with whose findings my own agree, all previous authors had found that respiration after vagotomy became apneustic, but attributed the result to removal of the posterior colliculi (Markwald and others) or to exclusion of the red nucleus (Henderson & Sweet). The complete removal of the posterior colliculi and red nucleus was confirmed in all my experiments by microscopic examination by Dr Una Fielding, of the Department of Anatomy, University College, London, to whom I have great pleasure in expressing my gratitude.

Intracranial division of the trigeminus nerve. That respiration continues to be regular after brain section at the level described, and bilateral vagotomy, might be due, according to the findings of Markwald, Asher & Lüsher and Loewy to rhythmical reflex inhibition from the trigeminus, similar to that of the vagus. This view of the trigeminus seems to have been accepted by Barcroft [1934], when he suggests that in order to smother the gasp into a normal respiratory act, a few afferent inhibitory influences out of all those normally reaching the centres are probably alone sufficient, especially those of the vagus and trigeminus.

In cats, under experimental conditions similar to those here described, Lumsden tried the effect of section of the trigeminus, but obtained results of doubtful meaning. In five out of six of his cases, typical apneustic respiration immediately developed, while in one case the respiration did not seem to suffer much, and continued regular.

In view of the importance of this point, it was desirable to repeat the experiments. Division of the nerve was carried out in twelve animals with the brain stem divided behind the posterior colliculi and the vagi cut. The nerves were crushed and divided close to their entrance into the pons. In three, after bilateral division of the nerves, respiration became rather irregular and eventually apneustic, but in all the other nine the double operation was well tolerated and did not cause any appreciable modification of the type of breathing, or of its rate and depth. It seems justifiable, therefore, to ascribe the alterations of respirations obtained after section of this nerve in some of my experiments and in those of previous observers simply to damage of the near parts of the brain stem, and not to the removal of a tonic reflex influence of the trigeminus on inspiration.

Section in the pons. While extirpation of all nerve tissue above the pons leaves the mechanism of quiet breathing to all appearances completely unaffected, a section falling in the pons, not higher than 2 or 3 mm. below its upper limit, leads, when combined with double vagotomy, to the classical development of apneustic respiration. A typical example is given in Fig. 1, where the brain stem had been cut by a section passing dorsally immediately behind the posterior colliculi, but reaching, ventrally, a level about 2·5 mm. lower than the superior border of the pons. It will be seen that, as long as the vagi were intact, respiration continued to be not appreciably different from normal, but immediately became apneustic when the vagi were blocked by a cold thermode. It was the same when the nerves were cut. It was immaterial whether the vagus was cut before or after the section in the pons.

So far, therefore, as the influence of the vagus is concerned, the present experiments do not agree with Lumsden [1923*b*], but do agree with all previous observers, in showing that as long as the vagi are intact, apneustic respiration does not occur even after section of the brain at this level, nor, indeed, at any level in the pons down to the superior limit of the striæ acousticæ.

It was seen above, that in animals breathing regularly after division of the vagi and section immediately above the pons, no tonic inhibitory influence of the trigeminus on inspiration could be detected. The same

negative result was obtained after vagotomy and sections of the brain stem at the level described here. The development of apneusis, in fact, was entirely independent of whether the trigeminus was cut or remained intact, as could be judged from the preservation of good corneal and nose reflexes.

Fig. 1. Abdominal respiration. Section of the brain stem along a plane passing dorsally immediately behind the posterior colliculi; and ventrally 2·5 mm. below the upper border of the pons. Between *x* and *z* the vagi were blocked by cold thermodes. In this and all subsequent figures read from left to right. Time: 2 sec.

Apneusis and afferent nerve roots

Having established the level at which the brain stem has to be cut in order to observe the phenomenon of apneusis, and confirmed the inhibitory influence of the afferent pulmonary vagus upon it, I proceeded to investigate how apneusis itself depended on influences coming from the periphery. If, in fact, the mechanisms here at work were the same as those responsible for the decerebrate rigidity, one would expect to see apneusis, like decerebrate rigidity, disappear, when all afferent channels have been cut off [Sherrington, 1898; Magnus, 1924; Pollock & Davis, 1931; etc.]. On the other hand, if apneusis is a gasp interrupted half-way by afferent inhibitory impulses set up in the periphery by the very act of inspiration, then removal of such inhibitory influences would result in the transformation of apneusis into gasping. To test the latter hypothesis, I endeavoured to make the deafferentation of the respiratory centre as nearly complete as I could in view of the possibility, already hinted at by Barcroft [1934], that if a few afferent channels

escape section, they might be sufficient to mask the effect of the abolition of all others.

Section of the dorsal (afferent) roots of the cervical and thoracic cord, and of the last four cranial nerves. After intercollicular decerebration, the spinal cord was exposed from the first cervical down to the 11th thoracic segment, below which it was divided. All dorsal roots above the section were then divided, from the first cervical pair to the 11th thoracic inclusive. This operation did not seem to alter respiration, which was

Fig. 2. Abdominal respiration. Intercollicular decerebration. Spinal cord cut below 11th dorsal segment. All dorsal roots of the cord cut above the level of section. Between *A* and *B* cooling of the vagi.

diaphragmatic before, and remained the same after the operation, continuing regular and rhythmical and at a normal rate (Fig. 2). These findings, therefore, disagree with those of Coombs [1918], who obtained a marked slowing of the respiratory rate after section of the dorsal roots of the cervical and thoracic cord. Blocking of the vagus, in such a preparation, results in slowing and deepening of breathing of the usual type and degree (Fig. 2).

When, as well as the dorsal roots, the phrenic nerves were also cut, in the neck, the costal respiration which resulted showed some small irregularities of rhythm and depth, and the cycle did not seem to proceed

Fig. 3. Costal respiration. Same conditions as in Fig. 2; in addition the phrenic nerves were cut in the neck.

so smoothly as the previous diaphragmatic respirations (Fig. 3). My findings, however, do not confirm Foá's observations of the importance for costal respiration of the integrity of the dorsal (afferent) roots of the cervical cord. In dogs and rabbits, Foá [1911] divided the vago-

sympathetic, and the phrenics, in the neck, and proceeded to cut the dorsal roots of the cervical cord. He noticed that "the costal respiration which continued regular although modified, after section of the vagi and phrenics, began to alter after division of the first dorsal roots, and when the last two (cervical roots) were cut it became profoundly altered, taking up that irregular type which it maintained after the subsequent sections", namely, "division of the cervical from the thoracic cord, and, later on, division of the bulb from the encephalon"—"The breathing

Fig. 4. Abdominal respiration. Intercollicular decerebration: dorsal cervica and thoracic roots and last four cranial nerves cut.

Fig. 5. Abdominal and costal respiration. Section of the brain stem across the pons. Dorsal roots of cervical and thoracic cord and IXth and XIIth cranial nerves cut. Between arrows the vagi were blocked by cold.

became slower and markedly irregular both in the number and amplitude of the respiratory acts. It was a respiration which could be called ataxic, like the movements of deafferented limbs. It was also an asthenic breathing since its depth was, on the whole, smaller than normal and because the respiratory muscles moved with difficulty, rising slowly, sometimes by jerks, often incompletely, falling back, exhausted, to the expiratory position." I always found that, whether the vagi and the phrenics had been cut or not, section of the dorsal roots, confined to the cervical cord alone, had no detectable influence on quiet respiration.

In animals with phrenics cut, Gesell & Moyer [1932] sometimes found no change in the type of costal respiration, after section of the last 5 cervical and the first 9 thoracic dorsal roots.

In Fig. 4 is given another experiment in which, in addition to section of the dorsal roots of the cervical and thoracic cord, as already described, the last four cranial nerves were cut at their exit from the skull. It will be seen that even this further interference with afferent impulses from the periphery had remarkably little influence on respiration. After a further section in the pons, a few millimetres below its superior border, combined with double vagotomy, apneuses ensued as usual. An example of this is seen in Fig. 5, in which after sectioning all dorsal roots of the cord, and the IXth and XIIth cerebral nerves, the pons was transected about 3 mm. below its upper border. When the vagus was cooled, apneusis immediately developed.

Fig. 6. Abdominal and costal respiration. Intercollicular decerebration. Dorsal roots of cervical and thoracic cord cut. It shows respiration dying down as a result of continued compression of the common carotids and vertebral arteries. Between I and II the arteries were released. II and III were taken 1 min., 30 sec. and 4 min. after I, showing the ability of the deafferented inspiratory muscles still to perform powerful gasps.

The same result was obtained if, in addition, cocaine was applied, beforehand, to the nasal mucosa so as to paralyse the trigeminal nerve endings.

If, after section of the dorsal roots of the spinal cord, acute anæmia was induced in the respiratory centres, by sudden and continued occlusion of the carotid and vertebral arteries, respiration became gasping, as usual (Fig. 6). Gasping was also obtained, as usual, after a low section, in the upper part of the medulla. The above experiments, therefore, show that deafferentation of the respiratory centres leaves the funda-

mental respiratory cycle practically unaltered—in particular it brings about, by itself, neither apneusis nor indeed gasping, these two modifications of respiration only occurring when in addition to deafferentation of the respiratory centres the usual experimental conditions for their production are obtained.

Intracranial section of the VIIth and VIIIth nerves

It was shown by Magnus [1924], Ranson [1929], Spiegel [1923], Pollock & Davis [1931] that the proprioceptive impulses from the muscles are not wholly responsible for rigidity after decerebration. According to Pollock & Davis [1931], for instance, the forelegs, after complete deafferentation, still show a marked rigidity, which is dependent on the position of the head and disappears only when the labyrinthine influences are abolished.

Very strong rigidity of the forelimbs after section of all the dorsal roots of the cervical and thoracic cord was also observed here, and its dependence upon the position of the head was fully confirmed. Therefore it was probable that in apneusis also the afferent influences still reaching the centres through the VIIth and VIIIth cranial nerves were responsible for its persistence under the experimental conditions described.

That the labyrinthine influences might play a part in the production of apneusis is suggested by the results of Lumsden [1923*a*] after intracranial section of the VIIth and VIIIth nerves, which "usually resulted in prolonged inspiratory spasms and convulsions, followed by gasping respirations alone, or gasping interspersed with a few incoordinated inspiratory spasms", in short, by such phenomena as he observed after section of the brain stem below the "apneustic" centre. Lumsden added that this did not happen when the VIIth and VIIIth nerves were destroyed from the external auditory meatus, and attributed the disturbances to compression of the brain stem by hæmorrhage and to the trauma by traction on the nerves. Since, however, he gives no details of the technique employed for the destruction of the nerve, from the external auditory meatus, it is not certain whether in this case also he destroyed the labyrinthine branch of the VIIIth, or left it intact instead, thus accounting for the negative results.

I repeated, therefore, the experiments of bilateral intracranial division of the VIIth and VIIIth nerves, after transection of the brain stem behind the colliculi. The cerebellum was retracted by smooth hooks, and the auditory nerves crushed in the manner already described. The operation was more difficult than that on the trigeminus, because of the special

liability to hæmorrhage; but the latter was soon overcome by plugging the region of the section with small swabs of cotton wool which were left permanently in place. Between the operation of one side and the other 10–15 min. were usually allowed to elapse.

The results were entirely negative. In no case did the spasms and gasps observed by Lumsden follow, and respiration continued quite regular. Nor did the operation interfere at all with the development of typical apneusis when later the pons was cut and the vagi divided. This was true whether the dorsal thoracic roots I–XI were intact or cut.

Attempts to find a relation between the position of the head and the development of apneusis, or its degree, in cats after section of the pons and vagi, with the VIIth and VIIIth nerves intact, were also all negative.

Discussion

A centre which is tonically inhibitory to inspiration, or, in accordance with the views of Henderson & Sweet, a centre which inhibits decerebrate rigidity of inspiratory muscles, is found to lie not in the posterior colliculi [Markwald, Asher & Lüscher, Lewandowsky, Trevan & Boock] or in the red nucleus [Henderson & Sweet], but in the upper region of the pons, as had been suggested by Lumsden [1923*a*]. Henderson & Sweet [1929], in order to cut the rubrospinal tracts, and thus prove that apneusis, like decerebrate rigidity, was dependent on the exclusion of the red nucleus, made a median longitudinal incision in the mid-brain, carrying it down as low as the upper region of the pons. It is, therefore, possible that the apneusis in their findings was due to the fact that with injury of the rubrospinal tracts they also damaged the centre inhibitory to inspiration in the superior region of the pons, or that they cut its connexions with the inspiratory centre, these connexions being probably crossed like the rubrospinal tracts.

Besides the difference between decerebrate rigidity and apneusis, namely, that the latter, unlike the former, does not arise after simple exclusion of the red nucleus, apneusis differs from decerebrate rigidity in its mechanism of production, because it is independent of proprioceptive impulses from the muscles involved, and from the other known sources of decerebrate rigidity, in particular, the labyrinthine influences.

In conclusion, there is between apneusis and decerebrate rigidity a superficial similarity, but the two phenomena are fundamentally different. In the light of our present knowledge there seems to be no reason for

regarding apneusis as anything else but an exaggeration of the inspiratory activity. This activity, according to Lumsden, was kept in check by the "pneumotaxic" centre, in the higher region of the pons. The present experiments, however, show that this is not the only mechanism, another being the tonic reflex inhibitory influence of the pulmonary vagus.

Deafferentation of the diaphragm has very little influence indeed on the strength and form of its respiratory cycle; deafferentation of the costal inspiratory muscles, on the other hand, makes their inspiratory contractions somewhat weaker and less smooth than normally. But the respiratory cycle remains fundamentally the same as in the intact animal. This was also true when the last four cranial nerves were severed in addition, so that the only remaining afferent channels still available were reduced to the Vth nerve, or to those fibres of this nerve which had escaped the action of the cocaine. In spite of this nearly complete deafferentation of the respiratory centres, regular respiration continued, while section in the pons caused the usual development of apneusis. If now one accepts Barcroft's theory of respiration, this would mean that the few afferent nerves still reaching the centres were sufficient very effectively to mask the absence of all the others. This is improbable, since the trigeminus, as shown in this paper, has not a tonic inhibitory action on respiration. Moreover, all available evidence shows that the effect on inspiration of inhibitory influences is in proportion to the number of fibres active and the degree of activity in each fibre. Such relation is seen in the action of the vagus, which is relative to the degree of expansion of the lungs, and in the effect on respiration of cutting only one vagus, or by crushing part of each vagus, when the inhibitory effects diminish in proportion.

The interpretation of the normal respiratory cycle, and indeed of apneusis, as reflex modifications of a fundamental gasp, brought about by inhibitory, moderating influences from the periphery, is not supported by experiment.

The results obtained here show that the automatic activity of the respiratory centres is not represented by the gasp, but by the form of the respiratory cycle which is seen after vagotomy in an intact animal. This conclusion finds further support in the work of Adrian & Buytendijk [1931] on the action currents of the brain stem of goldfish, completely isolated and removed from the body, when waves of potential change, almost certainly due to the persisting respiratory activity, were seen to be represented by large and slow curves, very similar to the usual respiratory cycle of the animals, and not by sharp and short discharges,

as would have been the case if their activity had now consisted of a series of gasps.

The results show in conclusion that apneusis represents the automatic activity of the respiratory centres, after excision of the upper part of the pons.

Summary

1. Respiration does not undergo any alteration if the posterior colliculi, or the red nucleus, or both, are cut off. After such extirpations vagotomy causes only the usual slowing and deepening.

2. If the section in the brain stem damages or cuts off the upper region of the pons, respiration continues rhythmical as long as the vagi are intact, but immediately becomes apneustic when these are divided.

3. Therefore, in the upper region of the pons there exists a mechanism (Lumsden's "pneumotaxic centre") which, apart from the vagi, rhythmically inhibits inspiration, thus producing regular respiration. This rhythmical inhibitory activity of the pneumotaxic centre is not due to afferent influences from the trigeminus nerve.

4. Apneusis, unlike decerebrate rigidity, is not dependent for its production on proprioceptive reflexes from the muscles of respiration or from the labyrinthine nerve. This, together with the fact that it is not brought about by extirpation of the red nucleus, disagrees with Henderson & Sweet's and Hess's contention that apneusis and decerebrate rigidity are two manifestations of the same fundamental process.

5. Regular, smooth, respiration and apneusis are still observable after such thorough deafferentation of the respiratory centres as would leave them still connected only with a few territories of distribution of the trigeminus. It is more reasonable, therefore, to regard them as expressions of the automatic activities of the centres concerned, rather than as the results of peripheral inhibitory influences, checking or interrupting a fundamental gasp.

The expenses of this research were defrayed by a grant from the Dixon Fund of the University of London, and I have pleasure in expressing here my indebtedness and gratitude.

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J. Physiol. (1938) 93, 24–38 612.843.14

THE THERMAL DECOMPOSITION OF VISUAL PURPLE

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(*Received* 25 *February* 1938)

In 1878 Ewald & Kühne measured the time necessary for solutions of visual purple to become decolourized completely when heated to various temperatures. They also investigated the effects of NaCl and of the absence of water on the thermal bleaching, and concluded that the reactions were very similar to the coagulation of albumen by heat. Since very little is known about the chemical nature of visual purple, it was decided to extend these observations by making some quantitative measurements of its thermal bleaching at various hydrogen-ion concentrations. Apart from the theoretical interest, it is of practical importance to know the amount of decomposition which may be expected during certain operations on the substance.

The experiments were carried out over the whole range of acidities within which visual purple is not very rapidly destroyed at room temperatures. The velocity of decomposition was followed by the progressive loss of optical density with time, and in what follows the term "decomposition of visual purple" means the loss of the typical colour since our only sure guide to the presence of visual purple is its characteristic wavelength absorption curve.

Theoretical

When visual purple solutions are bleached by light the colour changes can be expressed by assuming that an intermediate substance, "transient orange", is first formed which is rapidly decomposed at room temperatures to "indicator yellow" [Lythgoe, 1937]. Wald [1937] describes these changes in much the same terms. As its name implies the latter substance changes colour with the acidity of the solution, being pale yellow in alkaline and deep yellow in acid solution. Indicator yellow is

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unstable at room temperatures between about *p*H 3·5 and 6·0, but becomes progessively more stable in solutions of greater acidity or alkalinity. Visual purple is bleached by strong acids and alkalis, and the breakdown product has the same indicator properties and is indistinguishable spectroscopically from the product formed when it is bleached by light. The same also is true when visual purple is bleached by heat. This was shown by warming an alkaline solution (*p*H 9·56) to 46° C. for about 2 hr., by which time the original purple colour had been replaced by a very pale yellow. On making the solution acid the colour immediately changed to a deep yellow. The change was reversible.

The formation of indicator yellow complicates the determination of the velocity constant for the decomposition of visual purple, and still further complications are introduced owing to the fact that the indicator yellow is itself thermally unstable under the conditions of some experiments. Transient orange is very rapidly destroyed even at low temperatures. It is not known whether it is an intermediate stage in the thermal bleaching, but if so it must be destroyed so rapidly at the temperatures used in the present experiments that its formation will not be detectable. Since the decomposition of transient orange is very rapid we shall, in effect, be measuring the rate of decomposition of visual purple to the relatively stable indicator yellow.

The optical density D_t of a solution at any time, t, is the sum of the densities of visual purple, indicator yellow and impurities absorbing light. If the densities are expressed in terms of extinction coefficients and concentrations, the relation becomes

$$D_t = \alpha cl + \alpha' c' l + D_i, \qquad \text{.....(i)}$$

where α, α' are the extinction coefficients and c, c' the concentrations of visual purple and indicator yellow respectively, and l is the length of the cell; αcl and $\alpha' c' l$ are therefore the densities of the visual purple and indicator yellow respectively. D_i is the density of light-absorbing impurities.

The concentration of indicator yellow formed up to any time is z times the concentration of visual purple destroyed, where z is the stoichiometric relation between the two substances. If we call the initial concentration of visual purple, c_0, then $c' = z(c_0 - c)$. In this and what follows immediately it is assumed that the indicator yellow formed is stable.

Substituting $z(c_0 - c)$ for c' in (i)

$$D_t = \alpha cl + \alpha' z(c_0 - c)\, l + D_i. \qquad \text{.....(ii)}$$

In the final bleached condition $c = 0$. Let us call this final density D_f, therefore

$$D_f = \alpha' z c_0 l + D_i. \qquad \text{.....(iia)}$$

Substituting in (ii) and rearranging

$$D_i - D_f = c(\alpha l - z\alpha' l). \qquad \text{.....(iii)}$$

At the beginning of a period call the density D_0 and since the concentration of visual purple is then c_0,

$$D_0 - D_f = c_0(\alpha l - z\alpha' l). \qquad \text{.....(iv)}$$

If the decomposition of visual purple is a reaction of the first order, then

$$-\frac{dc}{dt} = k_1 c,$$

where k_1 is the velocity constant. Integrating and finding the constants

$$k_1 t = \log_e \frac{c_0}{c}. \qquad \text{.....(iva)}$$

Substituting the values of c and c_0 from equations (iii) and (iv), and eliminating $\alpha l - z\alpha' l$,

$$k_1 t = \log_e \frac{D_0 - D_f}{D_i - D_f}. \qquad \text{.....(v)}$$

If, on the other hand, the decomposition of visual purple is a reaction of the second order then

$$-\frac{dc}{dt} = k_2 c^2,$$

where k_2 is the velocity constant. Integrating and finding the constants

$$\frac{1}{c} - \frac{1}{c_0} = k_2 t \quad \text{or} \quad c_0 k_2 t = \frac{c_0 - c}{c}.$$

Substituting the values for c and c_0 from equations (iii) and (iv) and eliminating $\alpha l - z\alpha' l$,

$$k_2 t = \frac{D_0 - D_i}{D_i - D_f} \cdot \frac{1}{c_0}. \qquad \text{.....(vi)}$$

Up to this point it has been assumed that indicator yellow is stable. Under many conditions, however, this is not so and it loses its colour on heating. This is a further complication in determining the rate of thermal decomposition of visual purple, and it is necessary to apply a correction to the observed density readings in order to bring them to the values which would have been observed had the indicator yellow been stable.

The greatest absorption of light by visual purple is at 502 mμ. and the density falls off progressively towards 400 mμ. The absorption of light by indicator yellow, on the other hand, increases towards shorter wave-lengths. For each hydrogen-ion concentration there is a wave-length in the visible spectrum where the density of the indicator yellow

formed on bleaching is exactly the same as that of the parent visual purple. This is known as the cross-over wave-length. A series of absorption curves taken on a solution at different stages of bleaching would intersect at this wave-length provided that all the transient orange but none of the indicator yellow had been destroyed. At the temperatures used in the present experiments transient orange is very rapidly destroyed and any loss of density at the cross-over wave-length is a measure of the thermal destruction of indicator yellow. During an experiment, therefore, readings were taken at this wave-length concurrently with the readings of the loss of density at the maximum absorption of visual purple (502 mμ.). From previous work we know the densities of indicator yellow at different wave-lengths and for different acidities and it follows that we can calculate the loss of density at 502 mμ. due to the decomposition of indicator yellow knowing the decomposition at some other wave-length. For this purpose we first found the ratio of the density of indicator yellow at 502 mμ. to its density at the cross-over wave-length [Lythgoe, 1937]; we then multiplied the loss of density at the cross-over wave-length by this ratio. The resulting figure was then added to the measured densities of the solution at 502 mμ., thereby giving the density which would have been found had the indicator yellow been stable. These corrected values are those required in the equations.

Apparatus and preparation of solutions

The optical densities were measured by a photoelectric spectrophotometer [Bayliss *et al.* 1936]. The instrument was fitted with a new rotating shutter which reduced the time occupied by a single reading to 5 sec. and so made it possible to follow quicker changes.

Arrangements were made to keep the visual purple at a constant temperature during an experiment and to change this temperature rapidly between experiments. The optical cell containing the visual purple was placed in a hollow cell holder through which either heated or cooled water could be pumped. The water was delivered from a 2 gall. reservoir, the temperature of which was controlled by a thermoregulator of the expanding liquid type. It was fitted with a calibrated side arm and tap for the adjustment of the amount of mercury it contained and consequently of the temperature at which it regulated. A platinum wire was placed in the capillary of the regulator. As the temperature rose, the mercury made contact with the platinum and the current flowing in the closed circuit operated a "Sun-vic" relay. The relay switched off a heater in the reservoir and operated an electromagnetic clip which directed the

water returning from the optical-cell holder, through a cooling spiral before passing to the reservoir. When the temperature fell the contact in the thermostat was broken so that the heater was switched on and the water was returned direct to the reservoir. When temperatures below those of the surrounding air were needed, the heater was removed from the reservoir and the cooling spiral was immersed in an ice bath.

The temperature of the circulating water was measured by a thermometer situated near the cell holder. It was found that the thermostat maintained the temperature of the flow water to within about ±0·1° C. over the range 5–60° C. The time taken for the contents of the cell to reach a steady temperature and the relation of this temperature to that read on the thermometer in the circulating water were determined by means of a calibrated thermocouple immersed in the centre of the cell when filled with water. In the experiments readings were not begun until the visual purple was known to have attained a steady temperature. All figures for temperature have been corrected so as to show the actual temperatures of the visual purple. No special arrangements were made to stir the 0·4 c.c. of solution in the cell since it was thought that the frequent rotation accompanying a set of readings was sufficient to mix the liquid.

The solutions of visual purple were prepared from *Rana esculenta* by the method given in a previous paper [Lythgoe, 1937]. The retinæ were expressed, shaken with 0·6 p.c. NaCl solution and filtered through fine wire gauze. The filtrate containing the rods and pigment was centrifuged and the residue washed several times with 0·6 p.c. NaCl. The residue was then desiccated and extracted with petrol ether. After two washings with an acid buffer (*p*H 4·4–4·6) and one with 0·6 p.c. NaCl, the rods were shaken for 10 min. at 0° C. with 1 p.c. digitonin solution (1 c.c. for 12 frogs). After centrifuging strongly, the supernatant fluid was buffered by the addition of *M*/20 buffer solution [Clark & Lubs, see Clark, 1928] in equal volume. The final concentration of hydrogen ions in the solutions was measured by a Kerridge [1926] glass electrode used in conjunction with a valve electrometer [Dubois, 1930].

Conduct of experiments

For every preparation of visual purple the absorption curve was measured in the visible spectrum both for the unbleached solution and also after bleaching for a fixed time [Lythgoe, 1937]. At *p*H 4·2 to 5·57 the measurements were made with the solutions cooled to about 10° C. since above this temperature the destruction of indicator yellow is inconveniently rapid. The cross-over wave-length, the initial density at that

wave-length and the final light-bleached density of the solution (D_f) were given by these readings. The optical cell was then refilled; the heating circulation was turned on and after a suitable interval, readings of the density were taken over a period of at least half an hour. These readings were made alternately at 502 mμ. and at the cross-over wave-length, each reading being paired with a control. At lower temperatures where the destruction of the visual purple was slow, the same solution was used for the experiment at the next higher temperature, but otherwise a fresh sample from the same preparation of visual purple was used for each experiment. The velocity of the reaction was usually determined at four different temperatures for each preparation.

Results

Table I shows a typical set of readings at *p*H 6·83 and temperature 50·6° C. The method of correcting the results for the decomposition of indicator yellow is also shown. Column i gives the times at which the

Table I. Thermal decomposition of a visual purple solution at *p*H 6·83, showing method of correction for loss of indicator yellow. Temperature 50·6° C. Cross-over wave-length, 432 mμ. Initial density at 432 mμ., 0·282 (18·2° C.). Final density at 502 mμ. (D_f), 0·118 (18·2° C.). The figures in brackets are interpolated or extrapolated values.

i Time in sec.	ii Density 502 mμ.	iii Density 432 mμ.	iv Loss of density at 432 mμ.	v Loss of density at 502 mμ., i.e. col. iv × factor 0·37	vi Col. ii + col. v $= D_i$	vii $\frac{1}{t}.\log_{10}\frac{D_0-D_f}{D_i-D_f}$
0	0·379			[0·022]	0·401 (D_0)	—
94		0·220	0·062	0·023		
188	0·360			[0·024]	0·384	0·00014
280		0·215	0·067	0·025		
373	0·341			[0·025]	0·366	0·00015
467		0·212	0·070	0·026		
560	0·325			[0·026]	0·351	0·00015
653		0·209	0·073	0·027		
746	0·311			[0·027]	0·338	0·00015
847		0·207	0·075	0·028		
933	0·300			[0·028]	0·328	0·00014
1275		0·204	0·078	0·029		
1368	0·272			[0·029]	0·301	0·00014
1461		0·203	0·079	0·029		
1555	0·263			[0·029]	0·292	0·00014

readings were taken. Column ii gives the measured density of the solution at 502 mμ. at those times. Column iii gives the densities of the solution at the cross-over wave-length, whilst column iv shows the loss of density at that wave-length since the beginning of the experiment. From the previous paper we know that the ratio of the density of indicator yellow

at 502 mμ. to its density at the cross-over wave-length is about 0·37 in a solution of *p*H 6·83. The figures in column iv multiplied by 0·37 give the calculated loss of density at 502 mμ. (column v) the figures in brackets being interpolated values. In column vi the latter values have been added to the observed densities at 502 mμ. (column ii) to give the densities (D_t) which would have been observed had the indicator yellow been stable.

Table II shows a typical set of readings for conditions where indicator yellow is stable. The example chosen is *p*H 9·56, temperature 47·2° C.

TABLE II. Thermal decomposition of a visual purple solution in which the loss of indicator yellow is so small that correction is unnecessary. *p*H 9·56. Temperature, 47·2° C. Final density at 502 mμ. (47·2° C.) (D_f), 0·081. (Skeleton values only are shown.)

Time in sec.	Density 502 mμ. $= D_t$	$\frac{1}{t} \cdot \log_{10} \frac{D_0 - D_f}{D_t - D_f}$
0	0·316 (D_0)	—
31	0·307	0·00055
124	0·285	0·00049
216	0·265	0·00049
309	0·251	0·00046
402	0·237	0·00044
525	0·217	0·00045
628	0·207	0·00043
788	0·189	0·00043

The values of D_0, D_t and D_f were substituted in equations (v) and (vi) and both $\log_{10} \frac{D_0 - D_f}{D_t - D_f}$ and $\frac{D_0 - D_t}{D_t - D_f}$ were plotted against time to determine the order of the reaction. In Figs. 1 and 2 $\log_{10} \frac{D_0 - D_f}{D_t - D_f}$ has been plotted against time for two experiments. The results at *p*H 6·83, shown in Fig. 1, have been corrected for destruction of indicator yellow. No correction was necessary for the results at *p*H 9·56 shown in Fig. 2, since in solutions more alkaline than *p*H 7–8 the thermal decomposition of indicator yellow is negligible. The points fall very nearly on the straight lines which would have been obtained from a reaction of the first order. Nearly all the results show that the reaction proceeds too slowly towards the end of an experiment. The deviation can be seen in Fig. 2. Although the error in the logarithmic function is grossly disproportionate to the error in the measurement of density towards the end of an experiment when D_t and D_f are nearly equal to one another, the errors are consistently in the same direction. For most experiments, however, the results are in much worse agreement with the equation for a reaction of the second order (equation (vi)). It appears that the thermal decomposition of visual purple is a first-order reaction but that strict experimental verification is impossible owing to complicating factors.

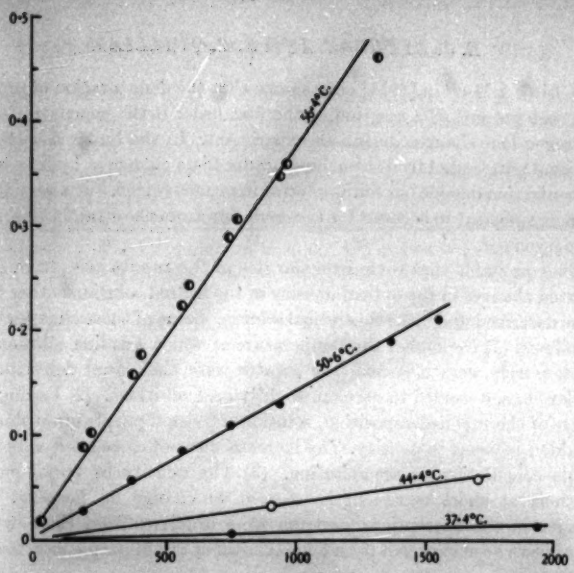

Fig. 1. Abscissæ: time in sec. Ordinate: $\log_{10}\frac{D_0 - D_f}{D_t - D_f}$. The values of D_t have been corrected for the fading of indicator yellow. The temperatures of the visual purple solutions are printed on the straight lines. For the experiment at 56·4° C. the figures of the ordinate must be doubled. *p*H 6·83.

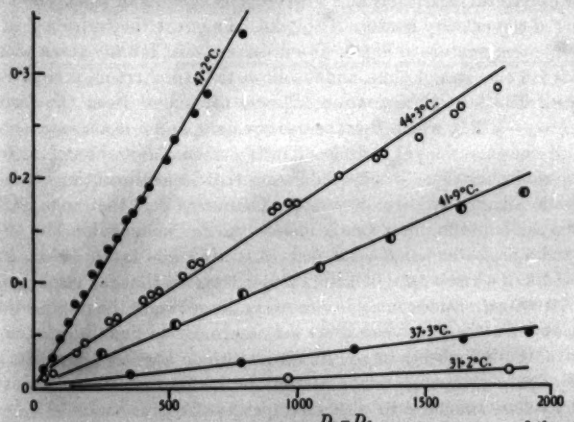

Fig. 2. Abscissæ: time in sec. Ordinate: $\log_{10}\frac{D_0 - D_f}{D_t - D_f}$. The temperatures of the visual purple solutions are printed on the straight lines. *p*H 9·56.

Chick & Martin [1911] have shown that the denaturation of protein does not proceed as a reaction of the first order if the concentration of hydrogen ions changes during an experiment. In the buffered solutions of visual purple used by us bleaching produced no change in hydrogen-ion concentration outside our limits of error in measurement, but a very slight change, sufficient to account for the deviation from the straight line, may have occurred.

It is probable that some irregularities in the results arise from complicated changes in the optical density of the heated solutions other than those described under the theoretical section. Some of these changes were as follows: (1) the higher the temperature at which alkaline solutions of visual purple were bleached, the greater were their final densities. A similar change seemed to occur in slightly acid solutions. (2) During the course of thermal decomposition, solutions of visual purple often showed a sudden increase in density. The increase was not associated with any visible coagulation or precipitation. (3) The density of visual purple solutions at short wave-lengths seemed to change on keeping. The changes in the absorption spectrum were different from those which would have been expected from a breakdown of visual purple to indicator yellow.

Graphs, similar to those in Figs. 1 and 2, were drawn for each experiment, and the slopes of the best-fitting straight lines were found. The derived velocity constants (k) are quoted throughout this paper on the basis of natural logarithms and with seconds as units of time. The logarithms of the velocity constants are plotted against the reciprocal of the absolute temperature in Fig. 3. It will be seen that for any given pH, the points fall on a straight line, and it follows that the Arrhenius equation is obeyed. The heat of activation, E, was calculated from the formula $\log_e k = c - E/RT$, where R is the gas constant and c is also a constant. For all concentrations of hydrogen ions the value of E is about 44,000 cal. per g.-mol., but there is some indication that in acid solutions it is less whilst in alkaline solutions it is more. This value is in the range 20,000–60,000 cal. found for most simple molecules. For hæmoglobin $E = 60{,}000$ cal. and for egg albumen $E = 135{,}000$ cal. [Chick & Martin, 1910]. From Ewald & Kühne's data, Wald [1935] calculated that for visual purple $E = 75{,}000$ cal. Some caution is necessary, however, in the interpretation of heats of activation when there are intermediate links in a chemical change, the links being in equilibrium with one another [Steinhardt, 1937]. Transient orange might possibly be such a link. The ratio of the velocity constants for a 10° rise of temperature (Q_{10}) is about 10 between

30 and 40° but somewhat less at higher temperatures. This value is very high compared with those for ordinary chemical reactions, but of the same order as those for reactions involving proteins.

Equation (v), which is that for a reaction of the first order, can be solved without knowing the concentrations of the solutions. To obtain the value of k_2 from the second order equation (vi), it is necessary to know c_0, a quantity whose absolute value is not yet known with any accuracy. By using D_0 in the equation instead of c_0, it is possible to obtain relative

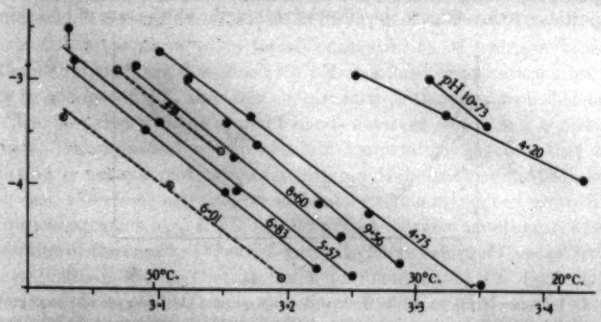

Fig. 3. Influence of temperature on the rate of decomposition of visual purple at various acidities. Scale of abscissæ: $1000/T$, where T = absolute temperature. Ordinate: $\log_{10} k$, where k is the velocity constant (time in seconds: natural logarithms). The upper dotted line is for a dialysed solution of low salt content; the lower dotted line is for a solution 2·0 *M* NaCl. The *p*H values of the solutions are printed on the lines.

values for k_2, and, using these values, E can be calculated. If the thermal decomposition of visual purple is a reaction of the second order, the calculated heat of activation is 53,000 cal.

It is quite possible that we are in reality dealing with a heterogeneous reaction, for it is well known that many reactions which at first sight appear to take place in solution are found on closer investigation to take place almost entirely on the walls of the containing vessel. In order to exclude this possibility the area of solution exposed to quartz was approximately doubled by adding quartz chippings to the quartz optical cells. In one experiment a solution of visual purple at *p*H 6·85 was prepared and the rate of decomposition at 44° C. was measured both with and without the addition of the chippings. The experiments were followed for one hour and in both the velocity constants of the reaction

were almost identical showing that an increase of surface exposed to the solution has no effect on the rate of the reaction.

Although the thermal bleaching of visual purple appears experimentally to be a homogeneous reaction of the first order, it does not follow that the mechanism of the reaction is unimolecular. If the mechanism were bimolecular and involved water, the concentration of the latter would remain constant during an experiment and the observed reaction would be of the first order. Reactions involving water are usually profoundly influenced by hydrogen-ion concentration and it is of importance to investigate the effect of this factor on the rate of bleaching.

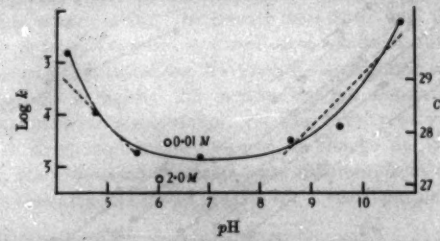

Fig. 4. Influence of pH on rate of thermal decomposition. Abscissæ: pH of visual purple solutions. Ordinate: the values of $\log_{10} k$ (Fig. 3) at 36° C. are given on the left. On the right are given the values of C in the approximate equation $\log_{10} k = C - 10{,}000/T_{abs.}$ Results from solutions with high (2·0 M) and low (0·01 M) salt content are indicated by open circles. For explanation of dotted line see text, p. 34.

The relation between pH and velocity constant at 36° C. is shown in Fig. 4. It will be seen that visual purple is thermally most stable at about neutrality, that it is fairly stable between pH 5·5 and 8·5, but that its stability falls off rapidly outside this range. It will be seen from Fig. 3 that the ratio between the velocity constants at different concentrations of hydrogen ions is approximately the same whatever the temperature at which comparison is made. It follows, therefore, that the relationship shown in Fig. 4 will also hold for other temperatures provided the velocity constants are multiplied by a factor. When graphs with the same co-ordinates as those used in Fig. 4 are plotted for reactions which are catalysed by water, the shape of the graph should indicate the roles played by the H^+, OH^- and undissociated H_2O in the reaction (see for instance Skrabal, 1927). If equal scales are used for ordinates and abscissæ, a reaction which is catalysed by H^+, OH^- and H_2O gives a graph with a horizontal portion due to catalysis by undissociated water

and with two side arms at 45° corresponding to accelerated catalysis as the number of H^+ or OH^- ions increases. It is possible that our results shown in Fig. 4 do fall on three straight lines but the side arms seem to be steeper than the dotted lines at 45° which would be required by the simple hypothesis. The reaction with H^+ and OH^- seems to be more nearly proportional to the square of the concentration of H^+ and OH^- ions.

The velocity of a reaction can be represented by the formula $k = PZe^{-E/RT}$, where k, E, R and T have the same definitions as have been used already, Z is the total number of collisions and P is the steric factor which is less than unity, $e^{-E/RT}$ is the energy of activation factor. Since E has the same value for all concentration of hydrogen ions it follows that the variation in k with pH at a fixed temperature must be due to changes in PZ, the term concerned with the effective collisions.

The fact that a single straight line is obtained when log k is plotted against the reciprocal of the absolute temperature suggests that the reaction is not composite (see for instance Hinshelwood, 1933), and one can, therefore, extrapolate without introducing serious error. The empirical equation $\log_{10} k = C - 10{,}000/T_{\text{abs.}}$ is approximately true, where the value of the constant C varies with pH. These values of C are given by the ordinates in Fig. 4. On calculation it is found that at 0° C. 1 p.c. of the visual purple would be destroyed in about 2750 hr. at pH 7, and in about 700 hr. at pH 9·6. These are useful figures to bear in mind in the dialysis of the solutions.

The addition of sodium chloride. Most workers since Ewald & Kühne have supported the view that visual purple is a protein. If this is so one would expect the thermal decomposition to be influenced by salt concentration. The experiments so far described were made on solutions having an effective salt concentration of about 0·025 M. Unfortunately the preparation of salt-free solutions of visual purple seems to be impossible since, when dialysed against pure water, the solutions become progressively more acid and the visual purple is bleached. The salt content can, however, be reduced. One solution was prepared by dialysing it in a cellophane sac against a 0·01 M buffer solution, pH 7·0, for 3 days in the dark at 0° C.: the final pH value of the solution was 6·18. The thermal decomposition of this solution was considerably faster than that of one of the usual solutions having the same concentration of hydrogen ions. The results of this experiment are given in Figs. 3 and 4. When, on the other hand, the NaCl content was increased to 2·0 M, the thermal decomposition was considerably retarded (pH 6·01). It was found, on the other hand, that for both the solution of high salt content and for the

solution of low salt content the heats of activation were the same as those of standard solutions of the same acidity.

The changes in thermal decomposition with salt concentration are similar to those found by Chick & Martin [1911] in their work on the denaturation of egg albumen and support the view that visual purple is either a protein or closely allied to one.

The thermal decomposition of indicator yellow. If indicator yellow is kept in solution more acid than about *p*H 3·5 or more alkaline than *p*H 6·5 it is only slowly decomposed at room temperatures (20°) or when warmed gently. An attempt was made to measure its thermal decomposition in the range of acidities where it is unstable but this was only partially successful on account of the variety of unknown reactions which have been described on p. 32. Two moderately satisfactory experiments were made, one at *p*H 4·75 and other at *p*H 6·83. The visual purple was first bleached by light under the standard conditions and the subsequent changes in the density of the indicator yellow were followed in the same way as for visual purple except that the wave-length used was different. For the solution at *p*H 6·83 a wave-length of 410 mμ. was chosen since this permitted both accurate and rapid records of the changes in density. For the solution at *p*H 4·75 readings were taken at

TABLE III. Velocity constants, k (time in seconds: natural logarithms) for the destruction of indicator yellow at various temperatures

*p*H 4·75		*p*H 6·85	
10·4° C.	$k = 1{\cdot}6 \times 10^{-5}$	27·6° C.	$k = 1{\cdot}0 \times 10^{-4}$
18·5	$= 1{\cdot}45 \times 10^{-4}$	44·6	$= 6{\cdot}4 \times 10^{-4}$
25·4	$= 3{\cdot}2 \times 10^{-4}$		
33·5	$= 1{\cdot}0 \times 10^{-3}$		

450 mμ., a wave-length which is near the maximum absorption of the acid form of indicator yellow. The results were calculated on the assumption that the thermal destruction of indicator yellow is a reaction of the first order. The greatest difficulty lay in the determination of the final density. The latter could not be determined by heating the solution until all the indicator yellow had disappeared since one or more of the unknown reactions already mentioned would probably occur. It was felt that the most satisfactory value was one which assumed that the solutions contained impurities similar to those described previously [Lythgoe, 1937]. The velocity constants for the temperatures investigated are given in Table III. At *p*H 4·75 the heat of activation was about 24,000 cal. This value was very little influenced by assuming different values for the

final density. The calculated heat of activation at *p*H 6·83 was 46,000 cal. but this value was obtained from velocity constants at only two temperatures and cannot be regarded as reliable.

Summary

1. The rate of decomposition of visual purple by heat has been measured at a variety of temperatures and concentrations of hydrogen ions. The reaction was followed by the loss of optical density with time.

2. When bleached by heat the product of decomposition, indicator yellow, appears to be identical with that formed by bleaching in light and by acids and alkalis.

3. Equations are developed to express reactions of the first and of the second orders when the product of bleaching (indicator yellow) is itself coloured. A method of correction for the destruction of indicator yellow is described.

4. The rate of thermal bleaching of visual purple solutions can be expressed best as a first order reaction. Some deviation occurs, but a closer approximation cannot be expected owing to complicated changes which occur in the solutions. The reaction is homogeneous and the chemical mechanism is either unimolecular or it is bimolecular with an excess of one reactant, probably water.

5. The Arrhenius equation is obeyed, giving a heat of activation of 44,000 cal. per g.-mol. This figure appears to be independent of the *p*H of the solutions.

6. The thermal decomposition of the solutions is accelerated by a decrease and is retarded by an increase in salt concentration.

7. The behaviour of visual purple solutions to heat is closely similar to that of protein solutions.

The authors wish to thank Dr C. F. Goodeve and Dr H. J. A. Dartnall for considerable help and advice. They also wish to thank the Rockefeller Foundation for a grant for work on the photochemistry of vision made in collaboration with Dr C. F. Goodeve.

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J. Physiol. (1938) 93, 39–60 612.741.13

THE AFTER EFFECTS OF A TETANUS ON MAMMALIAN MUSCLE

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(*Received* 25 *March* 1938)

Two accounts have recently appeared of the effects of an interpolated tetanus on the response of striated muscle to a series of regularly spaced, single motor-nerve volleys. Rosenblueth & Morison [1937] have recorded increases in twitch response as a result of tetani and a relief of partial curarization by the same means. Guttman and others [1937] have made similar observations on frog's muscle, and have concluded that the phenomenon is due to peripheral accumulation of a chemical mediator, which might be "acetylcholine or adrenine". As Rosenblueth and Morison point out, the long duration of the effects makes it improbable that acetylcholine should, in the mammal, be responsible for them, and they suggest that a mobilization of potassium ions offers a more reasonable explanation of the phenomenon.

In the present paper, the phenomenon of enhancement of twitch tension by a tetanus has been examined in detail, and evidence is advanced that it is due in part, at least, to potassium mobilization in the muscle fibre, and that the neuromuscular transmitting apparatus is not necessarily directly involved.

Methods

Cats decerebrated under preliminary ether anæsthesia were used in the great majority of the experiments. A few were done under chloralose anæsthesia, but we are under the impression that anæsthetics interfere to some extent with the phenomena under investigation.

Gastrocnemius, soleus and tibialis anterior have been chosen for recording. The tension was recorded by either a Sherrington torsion-wire myograph or a flat-spring myograph of similar characteristics. The methods used for close arterial injections have already been described

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(gastrocnemius, Brown and others, 1936; and Bacq & Brown, 1937; soleus, Brown, 1937*a*; tibialis anterior, Brown, 1938*a*). One modification has been introduced, the use of a box for maintaining the temperature of the muscle, and to prevent its drying when necessarily exposed for arterial injection and electrical recording. The box was of wood, sufficiently large to cover both hindlegs of the cat, electrodes, stands and injection syringe. The air contained in it was warmed and moistened by two carbon filament lamps, covered with tubular wicks which dipped into water.

The stimuli were applied through shielded silver electrodes, deeply buried between the thigh muscles, to the sciatic nerve, which had previously been firmly ligated at the origin of the hamstring nerve. Two pairs of electrodes were applied, the distal pair being used for the single shocks and the proximal pair for the tetani. The stimulating cathodes were placed distal to the anodes and were 1·8 cm. apart. When recording on the smoked drum, the intervals between single shocks were set at 10 sec. by a Lewis contact breaker, and, for the optical records, were given by a Lucas pendulum. The single stimuli were break shocks from a coreless induction coil, their strength being varied by a primary resistance. They were carefully chosen to be a little supramaximal. The tetanizing stimuli were obtained from a neon lamp oscillator, previously calibrated, and they were again slightly supramaximal. In some experiments single stimuli at intervals of 3 sec. were also taken from the neon lamp stimulator. It was found desirable to leave the preparation for ½–1 hr. after the dissection and fixation were finished, with electrodes in place on the nerve, to avoid spontaneous discharge and gross repetitive discharges of the nerve with single stimuli.

Action potentials were recorded from silver pins inserted in the tendon and belly of the muscle, usually 3–4 cm. apart. The tendon lead was connected to the input of two capacity-coupled amplifying stages feeding the four output pentodes of a Matthews oscillograph. The coupling condensers were of such a size as to give a fall to half value in 0·5 sec. of a rectangular input potential.

The electrodes used for recording demarcation potentials were two small calomel half-cells, filled with Ringer's solution and making contact with the tendon and belly of the muscle by two wool threads soaked in the solution, each completely encircling the muscle at the level of its application. The grid lead was from the musculo-tendinous junction, which was killed either by touching with a hot rod or by the application of a fragment of fused KCl [Bacq & Monnier, 1935].

Two methods were employed for measuring the demarcation potential. One, employing an electrometer valve and a "Cambridge" high sensitivity galvanometer, was used for visual measurements in conjunction with smoked drum recording. The killed end was led to the valve grid, and the anode circuit connected through a "backing off" circuit directly to the galvanometer. Some degree of drift was encountered, but this was checked before each actual measurement and the zero readjusted. In the other method we used a two-stage direct-coupled amplifier, consisting of two triodes arranged in a manner similar to the final stages of the amplifier described by Matthews [1935]. This was coupled to the pentodes of a Matthews oscillograph. With the relatively low amplification used, the highest giving 6·5 mm. camera deflexion for 1 mV., drift was only a fraction of a millivolt during the actual recording of an experiment. Calibrations were carried out regularly within the range of deflexion expected, before or after each record was taken.

Measurements of muscle temperature were made with an iron-constantan couple—a hypodermic needle with a central enamelled constantan wire—connected directly to a Moll galvanometer which was arranged to record on the sensitive paper used for the myographic responses. The cold junction was kept in ice in a vacuum flask and a balancing current was applied to maintain the galvanometer light on the recording surface. The sensitivity was such that changes of 0·01° C. could be measured with accuracy, the small size of the junction giving only a small heat capacity and corresponding rapidity of response.

In experiments where perfusion of the muscle was undertaken a double Dale-Schuster pump was used. A small cannula was inserted in the popliteal artery, the branches to gastrocnemius and the thigh muscles were tied and the perfusion localized to tibialis anterior, whose contractions were recorded, and the peroneal muscles. A Y tube with a 2-way cock was attached to the cannula and facilitated the change from one solution to another of different composition, contained in the second pump system.

The composition of the Locke-Ringer solution was as follows: NaCl 9·0 g.; KCl 0·42 g.; $CaCl_2$ 0·24 g.; $NaHCO_3$ 0·5 g.; dextrose 1·0 g.; distilled water to 1 litre.

Results

Effect of a tetanus upon the response of tibialis anterior to nerve stimulation

The typical effect of a tetanus upon the response of tibialis anterior to single maximal motor nerve volleys is an increase in the twitch tension. Fig. 1 illustrates such an effect. The nerve was stimulated by

single shocks at intervals of 10 sec. and at the signal, one such stimulus was omitted and replaced by a tetanic stimulation lasting for 2 sec. at a frequency of 57 per sec. At the end of the tetanus, the rhythmic stimulation was resumed. The twitch immediately succeeding the tetanus showed an increase in tension of some 70 p.c. beyond the previous level. From this summit the augmentation in tension gradually declines, but the whole effect may last as long as 10 min. This potentiating effect of a tetanus upon the single twitch tension is readily repeated in an experiment, provided sufficient time is allowed to elapse between successive

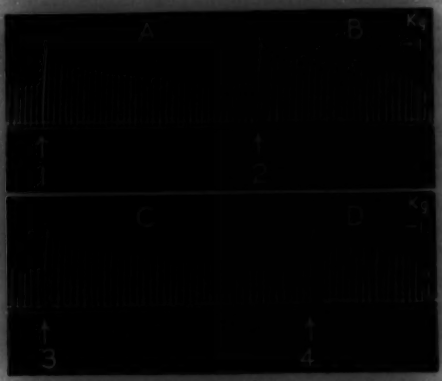

Fig. 1. Cat 2·7 kg. Decerebrated. Maximal motor nerve twitches of tibialis at 10 sec. intervals. At arrows, maximal tetani at 57 per sec. (1) for 2 sec., (2) 6 sec., (3) 10 sec., (4) 20 sec.

tetani. The first tetanus usually has the most striking effect, but subsequent tetani may produce a constant smaller change, which can be repeated with very little decline. This constancy of response has enabled us to determine approximately the optimum frequency for the production of the potentiation. With a duration of tetanus of 2 sec. the greatest potentiation was observed with frequencies of 50 per sec. and above, although the higher frequencies—above 200 per sec.—appeared slightly less efficacious than frequencies in the region of 50 per sec. The duration of the tetanus at a given frequency is of importance. With a stimulation frequency of 57 per sec. the effectiveness of the tetanus increases as its duration increases up to 6 sec. As the duration is increased beyond this, there is some fall in the potentiation of the twitch. With

duration greater than 10 sec. a change appears in the character of the response (Fig. 1). After short duration tetani the curve of the decline is characteristically concave upwards. After a tetanus of 15–20 sec., the declining curve shows a flattening, or a tendency to concavity downwards—a suggestion that an additional factor is complicating the effect.

The optically recorded twitch. Fig. 2 shows three maximal motor nerve twitches recorded with an optical isometric myograph; the first immediately before a tetanus of 6 sec., with a frequency of 45 per sec., the second, 15 sec. after the end of the tetanus, and the third 30 sec. after the end of the tetanus. This record has been chosen to show a potentiation of 50 p.c. and the associated changes in the duration of the twitch. The control twitch has a comparatively long relaxation

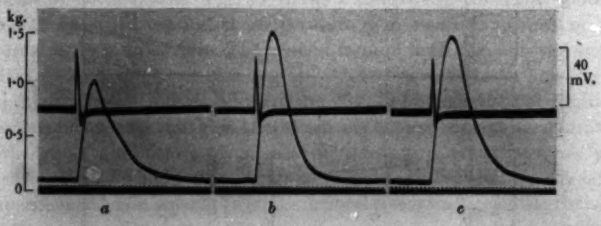

Fig. 2. Cat 2·5 kg. Decerebrated. Myogram and action potential of tibialis. Maxima motor nerve twitches (*a*) before, (*b*) 15 sec. after, and (*c*) 30 sec. after a motor nerve tetanus at 45 per sec. for 6 sec. Time 10 msec. A regularly recurring artefact has been removed from the electrical record.

time on account of the low temperature of the muscle. The first potentiated twitch shows a diminution in the time to the tension crest of 6 p.c. and a diminution of 4·5 p.c. in the time to half relaxation, in spite of the great increase in tension. As the twitches follow the tetanus at longer intervals, the time to half relaxation gradually lengthens, until it is a little (10 p.c.) greater than the "normal", and finally shortens again to its resting level as the potentiation passes off. The time to crest of the twitch, however, remains some 5 p.c. shorter than the normal for some 60 sec., and then returns to its previous level. This relative shortening of the total twitch time is probably to be associated with temperature changes in the muscle, but also makes it probable that, after the tetanus, the responses of the individual muscle fibres are at least as well synchronized as in the "normal" twitches—a point of importance in interpreting the changes in action potential which will be considered later. It is clear from the optical records that the recording of the twitch with

a mechanical lever possessing significant inertia will give rise to an exaggerated picture of the potentiation, on account of the inevitable increase in "overthrow" accompanying the quickened contraction. One result of this exaggeration is that, when a weak myograph is used for smoked drum records, an appearance of greater potentiation is obtained than in those taken under more strictly isometric conditions.

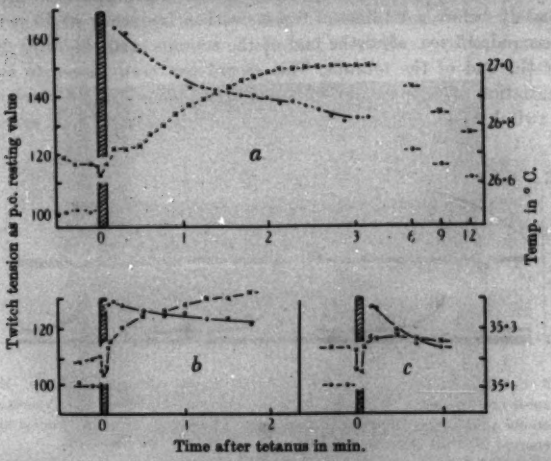

Fig. 3. Curves showing effect of tetanus at 57 per sec. on twitch tension (•) and temperature (×) of tibialis. (*a*) Muscle with low initial temperature, 6 sec. tetanus; (*b*) and (*c*) successive 4 sec. tetani in another experiment; (*b*) high initial temperature; (*c*) after occlusion of circulation.

Temperature changes. The observed changes in tension time suggested that the warming of the muscle by the tetanus might play a considerable part in the potentiation. Measurements of muscle temperature have, however, shown that there is no direct relationship between the rise in temperature and the increase in twitch tension.

Fig. 3*a* shows a typical temperature record taken from a muscle whose temperature was initially low (26° C.), because it had been suspended for some time in the vertical position without external heating. The immediate effect of the tetanus is a small fall in temperature. This fall in temperature we have observed in all our experiments, but we are doubtful of its significance. It is possibly attributable to vascular

changes, but it occurs so promptly with the increase in muscle tension that we are inclined to attribute it to an artefact due to movement of the needle thermocouple. In tetani of 2–4 sec. duration, the fall persists throughout the maintenance of tension. In more prolonged tetani (10–20 sec.) warming appears after some 5 sec., and, at the end of the tetanus, the muscle may be warmer than at the beginning.

From then onwards the temperature of the muscle gradually increases, continuing to rise after the end of the tetanus, and reaching its maximum in some 2–3 min. The absolute rise of temperature and its duration depend on a number of factors, such as external temperature, briskness of circulation and duration of tetanus. It is clear, however, from Fig. 3*a* that there is no obvious relation between the rise of temperature and the degree of potentiation. Fig. 3*b* shows a similar experiment in another animal, but with the initial temperature more nearly approaching the normal body temperature. The general form of both curves is the same, but Fig. 3*b* shows a smaller degree of potentiation. This is not due to the higher initial muscle temperature since, although the potentiation varies from one experiment to another, in any one experiment it is increased rather than diminished by increase in the resting temperature of the muscle. Figs. 3*b* and *c* are taken from two consecutive tetani in the same experiment 15 min. apart. In (*c*) the femoral artery was occluded proximal to the origin of the *profunda femoris*, so that the circulation through tibialis anterior was limited to the anastomatic channels from the gluteal vessels. Under these conditions there is only a very small rise in muscle temperature. The degree of potentiation, however, is equally great in two curves; they differ only in the duration of the potentiation, which, as might be expected, is less in the muscle with restricted circulation.

There is therefore no reason to suppose that the tetanic potentiation is to any important extent dependent upon gross temperature changes in the whole muscle.

The action potential. In tibialis anterior the potentiation of the mechanical responses to a maximal volley, after a tetanus, is characteristically associated with a diminution in the voltage of the action potentials, as recorded from belly to tendon (Fig. 4; see also Fig. 2). Only when the nerve volleys were submaximal has an increase of action potential been seen after the tetanus. The diminution is not great, seldom exceeding 10 p.c. of the previous level, but it follows closely the curve of increased tension (Fig. 5*a*).

We have been unable to detect any sign of the repetitive discharge of the muscle fibre, described by Rosenblueth & Morison [1937],

and we are inclined to attribute the repetitive discharge which they observe to repetitive firing of the nerve, at or near the point of stimulation.

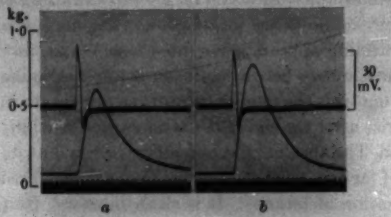

Fig. 4. Cat. Decerebrated. Myogram and action potential of tibialis. Maximal motor nerve twitches, (*a*) before and (*b*) 10 sec. after motor nerve tetanus at 46 per sec. for 4 sec. Time 10 msec.

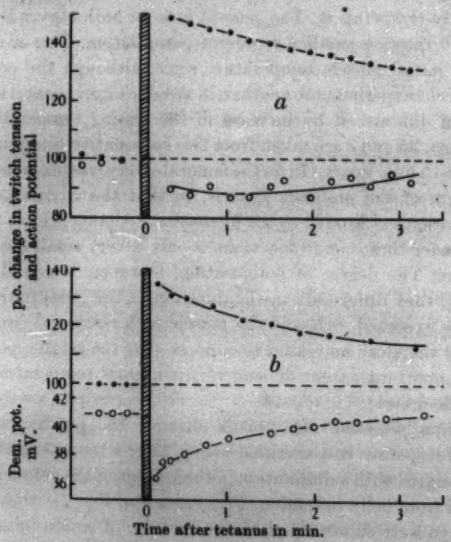

Fig. 5. (*a*) Curve showing effect of 6 sec. tetanus at 45 per sec. on twitch tension and action potential of tibialis. (*b*) Effect of 6 sec. tetanus at 57 per sec. on twitch tension (•) and demarcation potential (o) of tibialis.

If the stimuli used either for the tetanus or for the single volleys are very much supramaximal, the nerve apparently responds with more than

one volley, and small, isolated, single-unit twitches may be observed following the main response, a phenomenon especially well shown by concentric needle electrodes. Similar multiple responses have been described by Sherrington [1921] as occurring in mammalian muscle when supramaximal shocks are applied to the motor nerve. They can usually be avoided by reducing the stimulus strength until it is less than 50 p.c. supramaximal, and by leaving the preparation for 1 hr. after application of the stimulating electrodes, to allow the hyperexcitability of the freshly prepared nerve to subside.

One possible explanation of the diminished action potential after the tetanus would be a temporal scattering of the response of individual units, and a consequent asynchronism of the twitch response. Of this we have no evidence. The shape and duration of the action potential does not appear to be significantly altered, and the shortening of the time to the tension crest of the mechanical response is strongly against such an explanation. Another, and the most usual, cause of a diminution in the gross action potential of a muscle is a reduction in the number of fibres participating in the twitch; but it is not credible that a reduction of at least 10 p.c. in the number of active fibres should be associated with an increase in twitch tension amounting sometimes to 100 p.c.

This association of an increase in twitch tensions with diminution in the action potentials, after a tetanus, bears a striking superficial

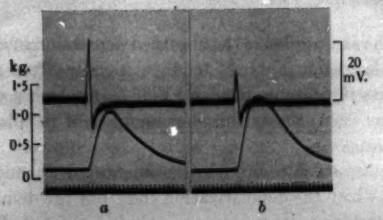

Fig. 6. Cat 2·7 kg. Decerebrated. Myogram and action potential of tibialis. Maximal motor nerve twitches, (*a*) before and (*b*) 15 sec. after close arterial injection of 2·5 mg. KCl in 0·25 c.c. Time 10 msec.

resemblance to the effects of arterially injected KCl previously described by one of us [Brown, 1937*b*] (Fig. 6). The effect of KCl on the action potentials was regarded as probably due to a depolarization of the muscle fibres. We have accordingly measured the demarcation potentials of muscles, before and after the potentiation of the mechanical, and depression of the electrical responses following injection of KCl or

tetanus. Arterial injection of KCl always caused a considerable diminution in the demarcation potential, its degree depending upon the amount given. With large doses, the demarcation potential may fall from 30 mV. to as little as 10 mV., but this change is accompanied by depression of the twitch tension. Smaller doses, 1–2·5 mg., reduce the demarcation potential by 5–20 mV. and this is accompanied by an increase or decrease in the twitch tension, according to the condition of the muscle and the number of previous injections. Similarly, tetanic stimulation was always followed by a prolonged fall in demarcation potential. Fig. 5*b* shows the diminution after a 6 sec. tetanus and the simultaneous increase in twitch tension. It is to be noted that the change in demarcation potential

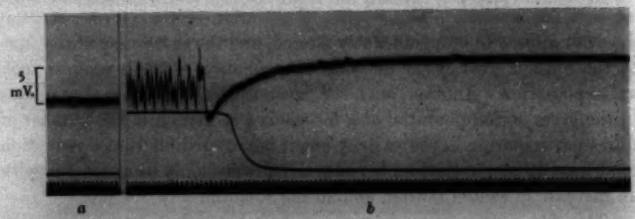

Fig. 7. Cat 2·5 kg. Decerebrated. Myogram of tibialis and demarcation potential recorded with D.C. amplifier. (*a*) Resting potential, (*b*) end of 4 sec. tetanus, showing development of depolarization. Time 10 msec.

(15 p.c.) is of the same order as the observed depression in action potential. The full depolarization appears to develop only after the muscle has relaxed from its full tetanic tension. Fig. 7, in which a quick recording instrument was used, shows this development of depolarization at the end of the tetanus.

It is difficult, on account of instrumental drift, to determine the exact point at which the depolarization ends, but the demarcation potential appears to return to its previous level before the end of the mechanical potentiation.

The effects of a tetanus on soleus

The effects of an interpolated tetanus on the single maximal twitches of the soleus muscle are quite different from those already described for tibialis anterior. Tetani of 2–4 sec. duration produce a depression of the tension of the first few succeeding twitches (Fig. 8A). The depression lasts for some 20–30 sec.; subsequent twitches are unchanged in tension. With tetani of 6–10 sec. duration there is a depression lasting for some

30 sec., but this may be followed by a small increase in the twitch tension. Tetani of 20 sec. duration may produce a very short lasting depression of the succeeding twitches, but this is sometimes absent and quickly passes into a potentiation, which may be as great as 60 p.c. and may last for 10 min. (Figs. 8 B and 9). The effects, however, are seldom as striking as those seen in tibialis.

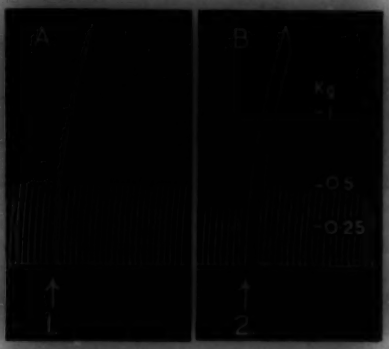

Fig. 8. Cat 3·2 kg. Decerebrated. Maximal motor nerve twitches of soleus at 10 sec. intervals. At arrows maximal tetani at 57 per sec. (1) for 2 sec., (2) for 20 sec. The lumbosacral sympathetic had been removed from this animal 4 weeks previously.

Electrical phenomena in soleus. The twitches succeeding tetani of 2 sec. duration or less are accompanied by action potentials showing somewhat irregular changes. The potential of a single response 8–10 sec. after the tetanus is always depressed about 20 p.c.; the subsequent twitches, still showing considerable reduction in tension, are accompanied by action potentials which vary 5 p.c. above and below the resting level.

The tetani of long duration, 10–20 sec., are always followed by a reduction in the action potential. Fig. 9 shows the plotted results of such an experiment. The great reduction of the first post-tetanic action potential and the rapid recovery of the second are characteristic in this muscle. It has been noticed in all the experiments on soleus that the action potential recovers its normal size well before the end of the mechanical potentiation.

The changes in demarcation potential follow closely the changes in action potential. There is an initial shift of some 10 mV. followed by

a relatively rapid return to the resting level. Although the initial change is greater, the diminution is of shorter duration and less constant than in tibialis.

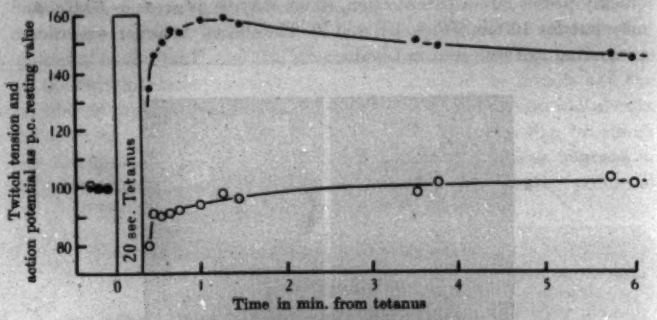

Fig. 9. Curve showing effect of tetanus at 50 per sec. on twitch tension (•) and action potential (o) of soleus.

Action of KCl *on soleus.* In tibialis anterior the concentrations of KCl which, when arterially injected, give the best potentiations are of the order of 10 mg. per c.c., but repetition of the injection is very liable to produce a profound and irreversible depression of the muscle. Gastrocnemius, on the other hand, is much less liable to permanent depression by KCl, even in concentrations of 100 mg. per c.c., and the same applies to soleus. But soleus differs again from gastrocnemius and tibialis in that it appears to be more resistant to the potentiating effects. Doses of KCl which are fully effective in tibialis produce only slight and transient potentiations in soleus. The effects of KCl on the demarcation potential of soleus are not significantly different from those described for tibialis anterior. Concentrations from 4–10 mg. per c.c. produce increasingly greater depolarizations, ranging between 5 and 20 mV. As in tibialis these may be accompanied by an increase or decrease in the twitch tension.

Effects of KCl *on the post-tetanic potentiation*

The arterial injection of KCl into tibialis anterior may be followed either by potentiation or depression of the motor nerve twitch according to the concentration given and the frequency at which injections are made. If a tetanus is given during a KCl potentiation, the post-tetanic potentiation appears as in the normal muscle (Fig. 10 A), and there is no significant change in the degree and duration of the effect. A higher

concentration of KCl causes a permanent depression of the motor nerve twitch and tetani during this depression produce either no change in subsequent twitches or even a slight further depression (Fig. 10 B).

Fig. 10.

Fig. 11.

Fig. 10. Cat 2·5 kg. Decerebrated. Maximal motor nerve twitches of tibialis. A, (1) Close arterial injection of 2·5 mg. KCl in 0·25 c.c., (2) 6 sec. tetanus at 80 per sec. Between A and B arterial injection 5 mg. KCl in 0·25 c.c. B (3) and (4) 6 sec. tetani at 80 per sec.

Fig. 11. Cat 3·0 kg. Decerebrated. Maximal motor nerve twitches of tibialis at 10 sec. intervals. A, 30 min. after start of perfusion with Ringer's solution. Between A and B injection into perfusion cannula of 30 mg. KCl. At arrows (1) and (2) motor nerve tetanus at 90 per sec. for 2 sec.

Experiments in which the muscle was perfused with Locke-Ringer solution have shown that in the early stages of perfusion the post-tetanic potentiation can be obtained, but the effect is small and rapidly diminishes as the perfusion proceeds, until, some 30 min. after the start, a tetanus produces only a depression of the subsequent twitches. Injection of additional KCl into the perfusion cannula at this stage evokes a re-appearance of a small degree of the post-tetanic potentiation (Fig. 11). The muscle under these conditions is sensitive to small excess of KCl, and repetition of the injection or increase in the K^+ content of the whole perfusing fluid may lead to a permanent depression and ultimate extinction of the muscle response.

Relation of the post-tetanic potentiation to the Orbeli phenomenon

In all the experiments so far described the stimuli were applied to the mixed nerve, and, consequently, fibres of the sympathetic system were stimulated with the motor nerve fibres to the muscle. It was possible, therefore, that the potentiation observed after tetanus might have been partly due to the effect of sympathetic stimulation, described by Orbeli. Certain features of the potentiation make such a supposition

unlikely; it is most conspicuous in a freshly prepared muscle, whereas the Orbeli effect is seen best after prolonged stimulation has led to severe fatigue; its onset is sudden after the tetanus, whereas the Orbeli phenomenon is usually described as developing more gradually and only after very prolonged stimulation. To settle the question, the lower abdominal sympathetic chain on both sides was removed aseptically, under ether, in three cats, from the level of the renal arteries to the last sacral ganglion, and degeneration allowed to proceed for 3–4 weeks. The tibialis and soleus muscles of these animals showed an effect of tetanus little, if at all, less than that observed in the muscle with the full innervation (see Fig. 8). We conclude that the Orbeli phenomenon plays, at the most a very subsidiary part in the post-tetanic potentiation.

Post-tetanic potentiation in denervated and fully curarized muscle

Guttman and others [1937] have ascribed the potentiation of twitch tension following a tetanus to accumulation of chemical neuro-muscular transmitter at the nerve ending. The absence of all evidence for a multiple response of the muscle fibre from our records, and the fact that the closely similar KCl potentiation can be seen in denervated and fully curarized muscle, led us to doubt this explanation of the phenomenon, and to determine whether the effect could be observed in directly stimulated muscle, in which the neuro-muscular transmitting apparatus had been excluded by degeneration of the nerve, or by curarine. This has proved to be the case. In Fig. 12A is shown the response of the directly stimulated gastrocnemius 6 days after section of the sciatic nerve, and in Fig. 12B the response of tibialis in which complete neuro-muscular block had been brought about by the arterial injection of 0·25 mg. of curarine. The effect in the latter case was not due to a "decurarizing" action of the tetanus, two maximal shocks to the nerve, interpolated at the blank space in the record, being without effect. It must be mentioned that the phenomenon in the denervated muscle is not well seen if degeneration is allowed to proceed beyond 10 days. Since, however,

Fig. 12. (A.) Cat 3·3 kg. Decerebrated. Directly excited twitches of gastrocnemius at 10 sec. intervals 8 days after section of sciatic nerve. At (1) tetanus at 50 per sec. for 2 sec. (B.) Cat 2·2 kg. Decerebrated. Directly excited twitches of tibialis at 10 sec. intervals after arterial injection of 0·25 mg. curarine. At (1), tetanus at 50 per sec. for 6 sec. At (2), stimuli applied to nerve.

the nerve ending is functionally excluded 72 hours after nerve section, the smallness of the effects observed after long denervation does not invalidate the conclusion that the integrity of the neuro-muscular junction is unnecessary for the production of the potentiation. Its decline or disappearance with longer degeneration is probably attributable to atrophic changes in the muscle fibres.

Effect of tetanus on neuro-muscular transmission

Wilson & Wright [1937] have recently shown that arterially injected KCl has the effect of relieving a more or less complete curare block in mammalian muscle. This effect we have been able to reproduce regularly. The mechanism of the action would appear to consist of a lowering of the

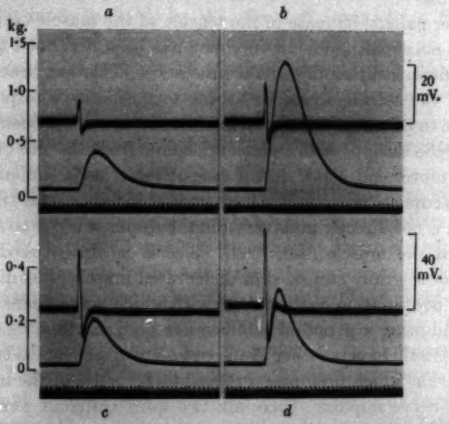

Fig. 13. Upper records, cat 2·7 kg., decerebrated. Myogram and action potentials of tibialis after intravenous injection of 1·0 mg. curarine. Maximal motor nerve twitch (*a*) before and (*b*) 15 sec. after close arterial injection of 2·5 mg. KCl in 0·25 c.c. Lower records, cat 3·6 kg., decerebrated. Maximal motor nerve twitch of tibialis after intravenous injection of 1·7 mg. curarine (*c*) before and (*d*) 0·4 sec. after motor nerve tetanus at 120 per sec. for 0·2 sec.

threshold of the muscle end plate to the stimulus transmitted from the nerve, since, in an incompletely curarized preparation, injection of small concentrations of KCl not only causes a many fold increase of the twitch tension, but also evokes a considerable increase in the size of the action potential (Fig. 13*a* and *b*). In view of the depressant action of KCl

on the action potential of the normal muscle with all its fibres responding, this can only mean that the K ions bring into action a large number of fibres to which the transmission of excitation by the nerve impulse had been blocked by curare. A brief tetanic stimulation of the motor nerve to a partially curarized muscle similarly has the effect of reinstating conduction temporarily at some of the blocked neuro-muscular junctions [Boyd, 1932]. Fig. 13*c* and *d* shows such an effect. The animal had received a dose of curarine sufficient to reduce the maximal motor nerve twitch to one-tenth of its previous value. The figure shows two maximal twitches, recorded before and after a tetanus at a frequency of 120 per sec. and lasting 0·2 sec. The increase, after the tetanus, in both tension and action potential is clear.

Discussion

In earlier papers, increase in the tension of the muscle twitch excited by a single maximal motor nerve volley has been described as resulting from the administration of several substances. This has been shown, in the case of the eserine series, to be due to a repetitive response of the muscle fibre to the single excitation from its nerve, and has been attributed to delay in the disappearance of acetylcholine from the site of its liberation at the motor end plate. In the case of veratrine, a similar repetitive response occurs in the muscle, but, in this instance, the transmitting mechanism is not directly involved, and the response is due to a repetitive tendency of the muscle fibre itself, since it is also evoked by direct excitation of the fully curarized or denervated muscle [Bacq & Brown, 1937]. The potentiation of the twitch with which we are here concerned, an effect following a period of tetanus, has been ascribed by Guttman and others [1937] to persistence at the end plate of a chemical transmitter, an explanation which presumes a repetitive response of the muscle fibre to a single nerve impulse. There are two quite different phenomena of post-tetanic potentiation; but neither, on our evidence, is due to repetitive response or to accumulation of acetylcholine.

(1) The potentiated twitch response of the normal muscle is the main subject of this paper. We have not only failed to find any signs of a repetitive response of the muscle fibre due to accumulation of acetylcholine; our evidence seems to us to be conclusive also against any direct concern of neuro-muscular transmission in the enhancement. A muscle, denervated by degeneration of its nerve, or one in which neuro-muscular transmission and the stimulating action of acetylcholine have been completely eliminated by curarine, when stimulated by directly applied induction shocks, shows the typical potentiation of its twitch responses

after an appropriate period of tetanus. We have failed to observe any indication of repetitive discharge in the potentiated response to nerve volleys, and we are inclined to suspect that the occasional repetitive responses described by Rosenblueth & Morison [1937] may have been due to the use of stimuli too greatly supramaximal, causing a repetitive firing of the nerve fibre. Temperature changes in the muscle do not run parallel to the potentiation, and cannot be responsible for more than a subsidiary effect. The phenomenon, it appears, must be due to some chemical change long outlasting the tetanus which evoked it, and imparting a higher tension to the single response of each fibre of the muscle. It is not due to repetitive response of the muscle to the single excitation from its motor nerve, or to the contraction of additional fibres through lowering of their end-plate threshold. That such a lowering of threshold does occur is made probable by the effects of a tetanus on the response of partially curarized muscle (*vide infra*); but it plays no part in the potentiation of the response of a normal muscle to a maximal nerve volley, since the electrical records show a diminution in the action potential accompanying such a potentiated twitch.

(2) The muscle *partially* poisoned with curarine shows an enhancement of the tension response to a single nerve volley, as a result of a preceding tetanus, which is easily confused with that seen in the normal or the *fully* curarized muscle, but has, in fact, a different origin and character. In partially curarized muscle, the responses of the muscle to maximal nerve volleys are submaximal on account of the failure of some of the muscle fibres to respond, because the threshold of excitability of their end plates has been raised too far by the curarine. Under such conditions a preceding series of nerve volleys in rapid sequence, though effectively transmitted to only a few of the fibres of the muscle, lowers the threshold at other end plates also for a subsequent period of some minutes, so that the maximal nerve volley now excites additional muscle fibres, as shown by the increase in the electrical response and the twitch tension. Adrian & Lucas [1912] and Bremer [1927] have shown that, in frog's muscle at a suitable stage of curare paralysis, a single nerve volley, though producing no visible response, may be succeeded by a short period of raised excitability of the end plate, so that a second suitably timed volley will excite the muscle to contraction. A similar effect in mammalian muscle has recently been described by one of us [Brown, 1938*b*], and the long-lasting restoration of neuro-muscular conduction by a preceding tetanus is apparently a similar phenomenon, the effects on the end-plate threshold of the nerve volleys in rapid sequence under-

going a summation, which greatly prolongs and intensifies their influence. Boyd [1932], who earlier described this effect in the mammal, attributed it to changes in the nerve impulses as a result of the previous, repeated activity. There is no ground, however, for postulating an effect on nerve fibres, or for supposing that the restoration of conduction is due to anything but a process of end-plate facilitation, whereby muscle fibres previously inexcitable undergo a lowering of threshold which brings them into activity. The tension evoked after the tetanus is probably also affected to some extent by the potentiation of the contraction of each responding fibre, like that seen in normal muscle. This effect is accompanied in normal muscle by a diminution in the action potential, and this diminution probably reduces the potential increase due to the response of additional fibres. The very large increase in potential gives a clear proof, however, that, under curare, the contraction of additional fibres is the predominating cause of the enhancement after a tetanus.

Both these different after effects of a tetanus can be closely simulated by a suitable injection of a potassium salt into the blood vessels of the muscle. Wilson & Wright [1937] have demonstrated the effect of potassium chloride, injected into the arterial stream at a distance from the muscle, in producing a temporary relief from the neuro-muscular block in a curarized muscle. We find that, in a partially curarized muscle, a suitable dose of KCl, injected into the artery of the muscle, during temporary arrest of the circulation, is followed by a recovery of the twitch tension and action potential evoked by a nerve volley, which is closely similar to that which follows a short period of tetanic stimulation of the nerve. One of us [Brown, 1937*b*] has shown that a similar injection of a potassium salt into a normal muscle produces a long persistent subsequent potentiation of the twitch response to direct and indirect stimuli; and here again we find that the effect is in all respects similar to that following a period of tetanus, the action potential being in both cases diminished, while the twitch tensions are increased.

These analogies are sufficiently complete to justify the suggestion that a change in the K^+ balance of the muscle fibre plays at least a part in the post-tetanic potentiation. Further support is given to this suggestion by our observation that, in the period during which the twitch tensions of the normal muscle are enhanced, either by injection of KCl or by a tetanus, the demarcation potential of the muscle is diminished, and that this change closely follows the potentiation of the twitch tension.

During recent years evidence has accumulated to show that there is a shift of the K ions of muscle during prolonged activity. Ernst &

Fricker [1934] have described an increase in the amount of diffusible K^+ in muscle after prolonged activity, and Reginster [1937] has described a similar alteration in ratio of free to combined K^+. The work of Fenn and his co-workers would indicate that this change in the state of muscle K^+ leads to an actual loss of K^+ from the muscle fibre, and these findings have recently been extended to mammalian muscle [Fenn & Cobb, 1936 and Fenn, 1937*a* and *b*]. If we picture the effects of injected KCl as being due to an excess of K^+ ions outside the muscle fibre surface, it is legitimate to associate the effects of a tetanus with a similar accumulation, derived from the interior of the fibre, where K^+ exists in far greater concentrations than in the blood or tissue fluids. Although injected KCl has a potentiating action, it has a further depressant effect, which is as frequently in evidence. This action offers an explanation for the failure, or even reversal, of the potentiating effect of a tetanus, when it is applied to a muscle which has previously been depressed by KCl.

The depression of the twitch response of soleus by a short tetanus, and the potentiation by a long tetanus, do not immediately fall into line with the observations on tibialis. It is suggestive, however, that we have regularly observed, as the immediate effect of an injection of KCl into soleus, a considerable but brief depression of the twitch, which is followed by a longer period in which the twitch may be slightly depressed, of normal tension, or potentiated. Fenn [1937*a*] has observed that soleus loses, during activity, more K^+ than the corresponding phasic muscle, tibialis. If this finding is viewed in conjunction with our observation, that a tetanus produces a greater change in demarcation potential in soleus than in tibialis, it suggests that the depressant action of a brief tetanus is attributable to a greater displacement of K ions in soleus. Whatever may be the true explanation of these effects, it is obvious that the muscle is very sensitive to changes of the K^+ balance within and without the muscle fibre, and that small changes may lead to either depression or potentiation of the twitch tension.

How far such a postulated movement of K ions can be correlated with the chemical events accompanying a tetanus is difficult to assess. Lehnartz [1931] has pointed out that the bulk of the formation of lactic acid resulting from a tetanus occurs after the cessation of activity, the acid so formed taking many minutes to disappear. The neutralization of this acid may be expected to lead to a displacement of the muscle K^+, bound to protein, and its mobilization in diffusible form. The gradual removal of the neutralized acid by oxidation and resynthesis would

permit the progressive recombination of K^+ in its bound form. If this were the only mechanism concerned in the mobilization of K ions in muscle contraction, we should expect that poisoning with iodo-acetic acid, by preventing the formation of lactic acid [Lundsgaard, 1930], would abolish the post-tetanic potentiation in normal muscle; and Guttman and others [1937] have found that, in fact, it does so. The available data do not make it possible even to form an opinion as to whether an increase of H ions *per se*, and apart from the displacement of K ions, plays any part in the potentiation. We know that K ions, artificially applied from without, will produce a very similar effect in the absence of acid formation. The suppression of the effect of a tetanus by iodo-acetic acid seems to indicate that, in default of acid formation, K ions are not liberated during activity in an effective concentration. We must be content, for the present, with the conclusion that the mobilization of K ions, probably secondary to acid formation, is an important factor in the post-tetanic potentiation.

Although the chemical changes accompanying muscular contraction may be responsible for the after effects of a tetanus in normal muscle, they can have only a small effect in the relief of curare paralysis. We have frequently observed that the tetanic stimulation of the nerve to a partially curarized muscle may itself produce a visible mechanical effect little greater than the previous response to a single nerve volley; yet such a tetanic stimulation may be followed by a long-lasting increase in the responsiveness of the muscle to subsequent single nerve volleys. Similarly, the facilitation which, under curare, follows a single nerve volley, may, in the frog's muscle, occur in the absence of any visible response to the first volley, while in mammalian and avian muscle [see Brown & Harvey, 1938] it has been shown to involve many more muscle fibres than those responding by contraction to the first nerve volley. It is obvious, therefore, that the facilitation process can develop at the end plate in the absence of propagated disturbances in the muscle fibre. A similar facilitation, in the absence of cellular discharge, takes place in ganglion cells [Eccles, 1936] and in the cells of the central nervous system, and is there accompanied by slow potential changes [Gasser & Graham, 1933]. There is, at present, no evidence that the facilitation process in curarized muscle is accompanied by slow potentials, since it is impossible to dissociate the prolonged post-tetanic depolarization and the slow potentials following single responses [Bishop & Gilson, 1927; Schaefer, 1936; Buchthal & Lindhard, 1937] from the contraction process proper. Though it is thus possible to distinguish the

facilitating effects of a tetanus from its effects upon the contractility of the muscle fibre, the close association of the two phenomena, and the ease with which both may be reproduced by K ions, make it not improbable that the facilitation also is connected with K^+, mobilized, in this instance, not by the contraction of the muscle fibre, but presumably in the motor end plate.

Summary

1. The after effects of brief tetanic stimulation, on the response to single, maximal motor nerve volleys of the tibialis anterior and soleus muscles of the cat, have been studied.

2. A motor nerve tetanus, of suitable duration, is followed by an increase of long duration in the twitch tension, which is independent of temperature changes in the muscle and the presence of its sympathetic innervation.

3. This potentiating effect of a tetanus can be observed in denervated muscle and in muscle completely paralysed by curarine, and is therefore independent of the integrity of the neuro-muscular transmitting apparatus.

4. The increase in twitch tension is accompanied by a diminution in the action potential and demarcation potential of the muscle, and is not associated with repetitive contraction of the muscle fibre.

5. The effects of a tetanus on the twitch tension, the action potential and the demarcation potential can be imitated by close arterial injection of KCl.

6. Neuro-muscular conduction can be restored, in a muscle partially poisoned with curarine, by a brief tetanus and by injection of KCl. These effects are due to a lowering of motor end-plate threshold.

7. It is suggested that the effects of a tetanus can be accounted for by a mobilization of K ions resulting from the chemical changes in the muscle fibre, which accompany contraction. The relief of curare paralysis is attributed to a similar mobilization at the motor end plates.

We wish to thank Dr R. B. Bourdillon for his advice and assistance in connexion with many of the electrical problems met with in this investigation.

Note added in proof. Since this paper was submitted for publication, a paper by Feng *et al.* (*Chin. J. Physiol.* 1938, **13**, 79), also dealing with post-tetanic potentiation in the mammal, has become available in this country. While on a number of points their interpretation of the phenomena, in the absence of electrical records, is different, they have independently reached a conclusion similar to ours, concerning the part played by mobilization of K ions.

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J. Physiol. (1938) 93, 61–73 612.492.8:612.352.1

THE INFLUENCE OF ANTERIOR PITUITARY EXTRACTS, INJECTED EITHER WITH OR WITHOUT INSULIN, ON THE GLYCOGEN CONTAINED IN THE LIVERS OF FASTING YOUNG RABBITS

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(*Received* 21 *March* 1938)

The observation of Houssay & Magenta [1925] that increased sensitivity to the hypoglycæmic action of insulin is produced by removal of the pituitary gland has been widely confirmed. Some investigators have claimed that the administration of anterior lobe extracts to normal animals induces a decreased sensitivity to the action of insulin in lowering the blood sugar, but some confusion has arisen because such extracts may also cause a substantial rise in the blood-sugar level of the injected animal [Houssay & Biasotti, 1931]. Cope & Marks [1934], however, found that injection of suitable anterior lobe extracts into rabbits might abolish the hypoglycæmic action of injected insulin without causing a rise of the blood-sugar level. Young [1936*a*] found that the pituitary factor which induced such insensitivity to the hypoglycæmic action of injected insulin was present in the prolactin fraction as ordinarily prepared, but could be differentiated from the gonadotropic and thyrotropic hormones and from prolactin [Young, 1937*a*]. The name "glycotropic factor" was suggested for the active principle [Young, 1936*b*] because the name "anti-insulin" or "contra-insulin" factor was unsatisfactory in the absence of knowledge as to whether or not all the manifold actions of insulin were inhibited by it. It must be emphasized that we do not yet know whether the glycotropic factor is or is not identical with one of the recognized anterior pituitary substances, though its identity with some has already been excluded.

That insulin promotes the formation of muscle glycogen from blood sugar has long been recognized, and Marks [1936] was able to demonstrate, using the eviscerated cat, that preparations of the glycotropic

factor inhibit this action. More recently Himsworth & Scott [1938] have shown that the glycotropic factor can inhibit the accelerating action of insulin on the fall of blood sugar in the hepatectomized rabbit.

Goldblatt [1929] made the interesting observation that the injection of insulin increases the glycogen content of the livers of fasting *young* rabbits, but not of adult animals. It was therefore of importance to know whether or not this action of insulin is inhibited by the glycotropic factor. Young [1937*b*] found that the amount of glycogen in the livers of adult rabbits receiving injections of the glycotropic factor during a short fast was much greater than in control animals receiving injections of inert protein. It was, therefore, of interest to know whether a similar effect could be observed in young rabbits, since young and adult animals apparently differ in some aspects of liver-glycogen metabolism.

Methods

Chemical. Blood sugar was determined by the Hagedorn-Jensen method, and glycogen by the method of Evans *et al.* [1931]. The moisture content of the liver tissue was determined by drying to constant weight at 100° C., the fat content of the dried material being estimated by means of exhaustive Soxhlet extraction with light petroleum. The nitrogen content of the fresh liver tissue was estimated by Kjeldahl's method. Although the value so obtained included the nitrogen present in materials other than protein, the amount of such material is small compared with the amount of protein, so the nitrogen content was multiplied by the factor 6·25 to give the approximate protein content of the fresh liver tissue.

All the anterior pituitary extracts used in the present investigation were prepared from fresh ox glands. A saline extract, and prolactin and thyrotropic fractions, were prepared at a low temperature by the methods described by Young [1938], the present "thyrotropic preparation" being the "non-prolactin-C" of the earlier publication. The thyrotropic preparation contained no detectable prolactin, as assayed by the pigeon crop gland test, while the prolactin fraction contained only traces of substances with thyrotropic or gonadotropic activities. Typical results of assays on these fractions have already been published [Young, 1938]. Crude saline extract and the thyrotropic preparation, but not the prolactin fraction, exhibited diabetogenic activity when administered daily in large amounts to normal dogs [Young, 1938]. It must be pointed out that the thyrotropic activity considered in this paper was determined by the method of Parkes & Rowlands [1934] and is therefore concerned with the increase in weight of the thyroid gland

of the immature guinea-pig, and not necessarily with an increase in secretory activity of the thyroid gland.

Biological. Young rabbits of Dutch or Himalayan stock, and weighing between 400 and 800 g., were obtained in litters, and the litter mates distributed among the various groups used. Each group usually consisted of 3–5 animals, and normally four groups were used in each experiment: (*a*) those receiving injections of an anterior pituitary extract and of insulin; (*b*) those receiving pituitary extract alone; (*c*) those receiving insulin alone; (*d*) those receiving no injection.

The diet consisted of oats, hay and cabbage, but food was withdrawn at midday on the day immediately preceding that of the experiment. To two groups an amount of extract corresponding to a total of 3 g. of fresh gland was given in two subcutaneous injections, one at 5.30 p.m. on the day before the experiment, and one at 9.30 a.m. the next morning. At 2 p.m. 1 unit of crystalline insulin was injected subcutaneously into the appropriate animals, and in some experiments the blood-sugar level of these rabbits was determined at 15 min. intervals for the next 2 hr. At 4 p.m. the first rabbit to be killed was given an intravenous injection of 1 c.c. of 10 p.c. amytal, which induced deep anæsthesia within 30 sec. The liver was rapidly removed and weighed, and samples taken for glycogen estimation, etc. In some experiments samples of the tibialis muscles were removed for determination of muscle glycogen. The animals were killed in succession, a member of group (*a*) being followed by one of group (*b*) and so on. The process of killing and weighing usually took about 30 min.

Results

The influence of anterior pituitary extracts on the hypoglycæmic action of insulin in fasting young rabbits

In Fig. 1 is shown the average blood-sugar response to 1 unit of insulin administered subcutaneously in each of three groups of young rabbits, one group receiving a crude saline extract of fresh gland, one receiving injections of the prolactin preparations, and the other not treated with pituitary extract. Each group consisted of three animals, and the blood-sugar curves represent the average result for each group. The curves show that, as with adult rabbits, two injections of crude saline extract of anterior lobe, or of a suitable preparation of prolactin, can abolish the hypoglycæmic response of young rabbits to insulin administered 4½ hr. after the last pituitary injection. The response to insulin of the group of animals receiving injections of the thyrotropic

preparation, which is not shown in Fig. 1, was diminished but by no means abolished, the blood sugar falling from an average of 0·115 p.c. to 0·080 p.c. during 2 hr.

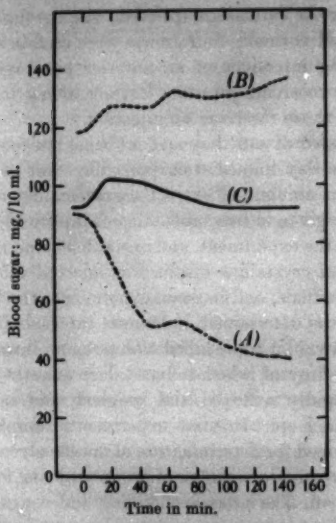

Fig. 1. The effect of 1 unit of subcutaneously administered crystalline insulin on the blood-sugar level of three groups of young rabbits fasted for 24 hr. Each curve represents the average response for a group of three animals. The insulin was injected at 0 min. A. Control animals. B. Animals receiving two injections of a crude anterior pituitary extract during the fasting period. C. Animals receiving two injections of a prolactin preparation during the period of fasting.

The influence of insulin on the liver glycogen content of young rabbits previously treated with anterior pituitary extracts

It was to be expected from previous results [Young, 1937*b*] that the glycogen content of the livers of young rabbits treated with anterior pituitary extracts during a 24 hr. fast would be substantially greater than that of control animals. Table I shows this to be so, and also demonstrates that the liver glycogen level is further raised by insulin injection only in those animals receiving a crude saline extract of fresh anterior lobe. In the groups of animals receiving the prolactin or thyrotropic

preparations the liver glycogen content is decreased rather than increased by the injection of insulin.

The results for the animals receiving pituitary extracts each represent the average figure for a group of eight animals, but the control group untreated with insulin consisted of seventeen young rabbits and that treated with insulin contained eleven animals. The values of the standard deviations of the mean results for the groups treated with crude extract indicate that injection of insulin induced a significant increase of liver glycogen. In the groups untreated with pituitary extract, insulin also caused a significant rise in the level of liver glycogen, but in the other groups it did not have this effect.

TABLE I. The influence of anterior pituitary extracts, and of anterior pituitary extracts +1 unit of insulin, on the liver glycogen content of young rabbits

Treatment during the fast previous to insulin injection	Liver glycogen content p.c.	
	Without insulin	With 1 unit of insulin
None	0·62 ± 0·25	2·24 ± 0·42
2 injections of crude saline extract	2·98 ± 0·24	4·62 ± 0·44
2 injections of prolactin preparation	2·14 ± 0·96	1·41 ± 0·28
2 injections of thyrotropic preparation	4·64 ± 0·99	3·36 ± 0·71

It was observed during the course of these experiments that the weight of the liver of the pituitary-injected animals was considerably greater than that of the control animals. The results are summarized in Table II. The results in this table are from the same groups of animals as those in Table I.

TABLE II. The influence of anterior pituitary extracts, and of anterior pituitary extracts +1 unit of insulin, on the average liver weight of groups of fasting young rabbits

Treatment during the fast previous to insulin injection	Liver weight as p.c. of body weight	
	Without insulin	With 1 unit of insulin
None	3·61 ± 0·11	4·05 ± 0·25
2 injections of crude saline extract	5·16 ± 0·62	5·15 ± 0·48
2 injections of prolactin preparation	4·36 ± 0·27	4·39 ± 0·24
2 injections of thyrotropic preparation	5·59 ± 0·29	6·00 ± 0·42

Insulin has no significant effect on the average weight of the liver of these groups of young rabbits. On the other hand, the livers of the

pituitary injected animals are considerably heavier than those of the untreated control animals, and it appears that the livers of the young rabbits receiving two injections of the thyrotropic preparation during a fast of 24 hr. may be more than 50 p.c. heavier than those of control animals. It should be mentioned that the mean body weights of the various groups were not significantly different, and the absolute weight of the liver was therefore proportional to the figure expressing liver weight as a percentage of body weight.

The total glycogen content of the livers of young rabbits treated with anterior pituitary extracts during a short fast

In Fig. 2 the mean percentage liver glycogen contents of the groups of control young rabbits and of those receiving pituitary extracts but no

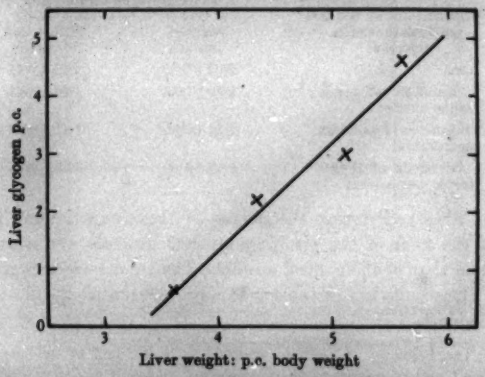

Fig. 2. The data for liver glycogen percentage content (Table I) plotted against liver weight expressed as p.c. of body weight, for the different groups of young rabbits.

insulin (Table I) are plotted against the mean liver weights of the corresponding groups (Table II). It is apparent that the percentage of glycogen present in these livers increases in linear relation with the liver weight, so that the total amount of glycogen in the livers of the pituitary-injected animals must exceed that in the livers of the control animals by a greater amount than would be suspected from the figures given in Table I. In Table III are given the mean total glycogen contents of the livers of those groups of young rabbits which received injections of pituitary extracts but no insulin. In the second column of this Table

are given the number of g. of glycogen present in the livers of the rabbits in the experimental groups, for each g. present in the livers of the control animals.

TABLE III. Total glycogen content of the liver of young rabbits treated with anterior pituitary extracts during a fast of 24 hr.

Treatment during the fast	Total mount of liver glycogen, g. per 500 g. rabbit	Glycogen present in the livers of the experimental animals, per each g. in the control livers g.
None	0·12 ± 0·05	1·0
2 injections of crude saline extract	0·91 ± 0·14	7·6
2 injections of prolactin preparation	0·55 ± 0·33	4·5
2 injections of thyrotropic preparation	1·37 ± 0·40	11·4

The livers of the animals receiving the thyrotropic preparation contain over eleven times as much glycogen as the livers of the untreated animals.

The general chemical constitution of the livers of young rabbits receiving injections of pituitary extracts during a short fast.

It is clear from the data given in Tables I and II that the greater weight of the livers of the experimental animals, as compared with that of the control animals, was by no means accounted for by their greater glycogen content. It was, therefore, of interest to determine to what constituents of the liver tissue this extra weight was due.

For this purpose experiments were carried out similar to those previously described, but including analyses of the liver tissue for water, nitrogen and fat contents, as well as for glycogen. From the data so obtained it was possible to determine the amounts of protein,[1] fat, glycogen and water present in 100 g. of the liver of control animals, and to compare these quantities with those for the similar constituents present in the increased amount of liver tissue which, in the experimental group, corresponded with 100 g. of liver in the control animals.

Table IV gives the results for an experiment of this kind in which a thyrotropic preparation was used. In this instance the livers of the experimental group were, on the average, 37 p.c. heavier than those of the control group.

It will be seen that protein, glycogen, fat and water together constitute about 97 p.c. of the liver tissue in each case, and that the total

[1] The figure for protein includes other nitrogenous substances calculated as protein.

Table IV. The amount of the different constituents of liver tissue present in corresponding amounts of control and experimental liver

Constituent	Amount present in 100 g. of control liver g.	Amount present in 137 g. of experimental liver g.
Protein*	18·5	17·4
Glycogen	2·9	8·2
Fat	3·8	18·4
Water	72·1	89·0

* The figure for protein includes other nitrogenous substances calculated as protein.

amount of protein present in each liver is approximately the same. The increase in liver weight is therefore not due to a true "hepatomegaly", but to the presence of extra water, fat and glycogen, extra water accounting for nearly half of the difference in weight of the liver in the two groups. Similar results were obtained in experiments with the other pituitary fractions. The prolactin and thyrotropic preparations appeared to be similar as regards their power of inducing an increased fat content in the livers of these fasting young rabbits.

The influence of anterior pituitary extracts on the muscle glycogen content of fasting rabbits

Muscle glycogen estimations were not made in all the experiments on young rabbits, but in those instances in which they were, the muscle glycogen content of the pituitary-treated animals was significantly greater than that of control animals, as shown in Table V.

Table V. The influence of anterior pituitary extracts on the muscle glycogen content of fasting young or adult rabbits

Type of rabbit	Treatment during the fast	Muscle glycogen content g. p.c.
Young	None	0·76 ± 0·03
	2 injections of prolactin preparation	0·96 ± 0·06
	2 injections of thyrotropic preparation	0·90 ± 0·01
Adult	None	0·55 ± 0·06*
	2 injections of prolactin preparation	0·83 ± 0·06*
	2 injections of thyrotropic preparation	0·88 ± 0·14

* Data from Young [1937*b*].

The data given by Young [1937*b*] for muscle glycogen values from experiments with adult rabbits are included in Table V because standard

deviations of the mean values were not given in the earlier communication. The figures given in Table V show that the differences in muscle glycogen content in the earlier experiments are highly significant.

It may be remarked here that one of us (H.P.M.) has observed very high muscle glycogen values in spinal, eviscerated cats which had received injections of the prolactin preparation for 2 days. These animals had fasted for 16 hr. before the samples were taken for muscle glycogen determination, in connexion with another investigation. The average muscle glycogen percentage for a series of twenty cats injected with the prolactin preparation was $1{\cdot}29 \pm 0{\cdot}08$, compared with a value of $0{\cdot}77 \pm 0{\cdot}05$ for the same number of controls.

Discussion

The results of the present investigation confirm the observation of Young [1937*b*] that rabbits receiving suitable injections of the prolactin preparation during a short fast have higher liver and muscle glycogen levels than control animals. Unpublished observations on adult rabbits have shown that similar results could be obtained with the thyrotropic preparation, and the results in the present paper show that they can be obtained with young rabbits also. The greater weight of the livers of the pituitary-injected animals causes the total liver glycogen content of the experimental animals to exceed that of the control animals (Table III) by a much greater margin than would be suspected from the percentage content of glycogen in the livers (Table II). It is probable that the same applied in the earlier experiments [Young, 1937*b*], but the total weight of the liver was not determined.

The condition of our injected, fasting rabbits is, in many ways, the opposite of that of hypophysectomized animals. It is generally agreed that, during a short fast, hypophysectomized rabbits lose their reserves of glycogen more rapidly than normal animals, and that they are extremely sensitive to the hypoglycæmic action of insulin. The recent experiments of Cope [1937] on hypophysectomized young rabbits, strikingly illustrate these points. Our injected young rabbits have very large stores of glycogen after a short fast, and are completely or relatively insensitive to the hypoglycæmic action of insulin.

Russell & Bennett [1936] and Fisher *et al.* [1936] have shown that hypophysectomized rats lose muscle and liver glycogen at an abnormally high rate during a short fast, and that the abnormal loss of muscle glycogen, but not of liver glycogen, can be prevented by treatment with a crude anterior pituitary extract during the fast. These workers suggest

that the crude anterior lobe extract they used contains a "glycostatic factor" which depresses carbohydrate oxidation. In hypophysectomized rats the absence of this factor is considered to be the cause of the increased loss of carbohydrate during fasting. The question whether or not the active principle which influences the liver and muscle glycogen contents of intact fasting animals, in our experiments, is identical with the "glycostatic factor" influencing the muscle glycogen content of hypophysectomized animals, cannot be settled at present, although there appears to be no obvious reason to suggest that the two factors are different. According to Russel, [1938] the "glycostatic factor" cannot be identical with the growth, adrenotropic, gonadotropic, lactogenic or thyrotropic substances. Our substance is probably not the gonadotropic or thyrotropic hormone, or prolactin, as it is present in prolactin preparations which have virtually no thyrotropic or gonadotropic activity, and in thyrotropic preparations which are free from prolactin. According to the present findings the thyrotropic fraction is more active in influencing the glycogen content of fasting rabbits, per g. of fresh gland extracted, than is the prolactin preparation, whereas the prolactin has substantially more glycotropic activity than the thyrotropic fraction. This suggests that the glycotropic factor, and the factor determining the amount of glycogen stored during a short fast, may be distinct entities, but a more satisfactory separation would be required before a definite conclusion could be drawn. The anterior pituitary factor inducing increased fat storage in the liver [Best & Campbell, 1936] appears to be present in both the prolactin and thyrotropic fractions.

Our results show that the rise of liver glycogen which usually follows the administration of insulin to fasting young rabbits is prevented by the injection of two doses of the prolactin or of the thyrotropic preparation, during the preliminary period of fasting. The interpretation of this result is complicated by the fact that the initial liver glycogen content of the pituitary-injected animals is so much more than that of the control animals. It might be suggested that insulin fails to raise the liver glycogen content, not because the pituitary extract exerts a specific inhibition of this action of insulin, but because the liver glycogen level is already so high that a "homeostatic" mechanism retards any further increase. The slight fall of liver glycogen level which insulin caused in these experiments would be in keeping with this idea. On the other hand, in those young rabbits which received the crude pituitary extract, the rise in the percentage of glycogen in the liver under the influence of insulin was similar to that in control animals. The lack of rise of liver

glycogen under the influence of insulin, in the animals receiving injections of the prolactin and the thyrotropic preparations, cannot therefore be ascribed merely to the high initial level. It is not clear why the results with these fractionated preparations should be different from those with the crude anterior lobe extract.

The question of the source of the glycogen which accumulates in the livers of young rabbits under the influence of insulin has been discussed at some length, but no general agreement has been reached [see Goldblatt, 1936]. The source of the extra liver glycogen present in the pituitary-treated animals, uninjected with insulin, is also not clear. Muscle glycogen cannot provide the source, as this appears to increase as well as the liver glycogen in our experiments. Russell & Bennett [1936], as indicated by their naming the active principle the "glycostatic factor", believe that the greater glycogen content of the pituitary-injected animals in their experiments, is due to a decreased utilization of carbohydrate during the fast. The possibility that the evidence might also be interpreted as indicating increased glyconeogenesis from fat during the fast was indicated by Young [1937*b*].

The fact that glycogen and fat are present together in such large amounts in the livers of the injected animals is of interest as providing a clear exception to the so-called Rosenfeld's law [1903], according to which liver glycogen and liver fat are mutually antagonistic and vary in an inverse manner. Kaplan & Chaikoff [1936], and others, have also observed exceptions to this "law". The simultaneous presence of large amounts of fat and glycogen in the livers of the injected young rabbits is associated with an increased total amount of water in the liver, although the percentage of water in the liver tissue is decreased. The fact that the total amount of nitrogenous material in the liver is little altered by the injections suggests that there has been no growth of the liver cells or "hepatomegaly". Best & Campbell [1938] have also observed an increase in liver weight in fasting animals receiving injections of anterior pituitary extract. The increase appeared to be due mainly to the deposition of fat and water. Glycogen was not estimated but a slight increase in non-fatty solid material was found.

Kaplan & Chaikoff [1936], as the result of extensive studies on dogs, concluded that the storage of glycogen or of fat in the liver is not accompanied by the simultaneous deposition of measurable amounts of water, but that the total water content of the liver tissue is directly proportional to an analytical fraction, the chief constituent of which is protein. In our experiments, however, the total amount of nitrogenous

material in the livers of the injected rabbits was similar to that in the livers of the control animals, but nearly 50 p.c. of the difference in the weights of the livers was due to an increase in the total amount of water present in the livers of the experimental animals. The rest of the difference was accounted for by the greater glycogen and fat contents of the livers of these animals. It seems certain that the deposition of either fat or glycogen, or both, was accompanied by the deposition of water in the liver tissue in these experiments.

In conclusion we wish to stress the fact that, in the absence of information as to the glycogen content of the animals at the beginning of the fast, we have, as yet, no evidence as to whether or not the glycogen content of the pituitary-injected animals rose during the 24 hr. fast. Non-fasting animals are liable to vary so much in glycogen content that a definite statement cannot be made on this point at present. We are able to state, however, that the administration of anterior lobe extract to a rabbit during the second 24 hr. of a 48 hr. fast has so far failed to demonstrate an increase in total glycogen content.

It should also be pointed out that our experiments were completed within about 24 hr. Similar results will not necessarily be found if injections of pituitary extracts are given for longer periods [cf. Bennett, 1937].

Summary

1. Young rabbits, as well as adult animals [Young, 1937*b*], receiving two injections of suitable glycotropic anterior pituitary extracts during a 24 hr. fast, exhibited substantially higher liver and muscle glycogen levels than did control animals fasting for a similar period.

2. The subcutaneous administration of 1 unit of insulin to fasting young rabbits, treated during a 24 hr. fast with a suitable thyrotropic or prolactin preparation, was not followed by a rise of the liver glycogen level. Young rabbits treated with a crude saline anterior lobe extract during the fast, exhibited a high level of liver glycogen which was raised still further by the injection of 1 unit of insulin. The reason for the different effects of the crude gland extract, and of the fractionated products, is not clear.

3. As well as containing a greater percentage of glycogen, the livers of the pituitary-injected animals were substantially heavier than those of control animals, the increase in liver weight being proportional to the increase in glycogen content. The total amount of glycogen present in the livers of the experimental animals was therefore much greater than that of control animals. The livers of the young rabbits receiving

injections of the thyrotropic preparation during the fast contained over eleven times as much glycogen as the livers of uninjected, control animals.

4. The greater weights of the livers of the pituitary-injected young rabbits was due to the presence of extra total water, fat and glycogen, as compared with the livers of control animals. Extra water accounted for nearly one-half of the difference in weight. The pituitary extracts did not produce true hepatomegaly under the conditions of our experiments.

5. Our factor, which influences the glycogen levels of fasting, intact rabbits, may be identical with the "glycostatic factor" of Russell & Bennett, which retards the utilization of muscle glycogen in fasting, hypophysectomized, young rats. It seems probable, from the relative activities of different fractions, that our factor is not identical with prolactin or with the gonadotropic or thyrotropic hormones. There is some evidence to suggest that it is not identical with the glycotropic ("anti-insulin") factor, but no definite conclusion can yet be drawn.

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J. Physiol. (1938) 93, 75–80 612.119:611.133

THE CAROTID SINUS AND BLOOD REGENERATION

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(*Received* 18 *October* 1937)

In a previous communication [Latner, 1937] it was shown that, in the rabbit, removal of both carotid sinuses and denervation of the aortic arch gives rise to an altered blood picture. Only a few experimental animals were used and no controls were observed. It was, therefore, deemed necessary to repeat the work using a further small number of animals, and performing suitable control experiments.

In the previous work, the presence of megaloblasts without normoblasts seemed rather strange. In fact this apparent discrepancy might lead to some criticism. At the time that this work was carried out, the change in the blood picture was not expected. No examination of the cells was made until some time after the operation. It was, therefore, considered of importance to follow the blood picture in greater detail. In this more detailed investigation, the presence of normoblasts as well as megaloblasts has been amply demonstrated.

Methods

The operative procedure adopted was exactly the same as in the previous communication, except that immediately after ligature of the internal carotid, and just before proceeding to remove the portion between the ligatures, the common carotid and the internal and external carotids were treated with a strong solution of phenol, applied on a tiny piece of cotton wool held in a fine pair of Spencer Wells forceps. The last inch of the common carotid and as much as possible of the other two arteries were treated in this manner. The phenol was removed by swabbing with cotton wool soaked in alcohol, and the alcohol rapidly removed by washing with sterile water.

This procedure was adopted in order to make sure that the arterial regions were completely denervated. The nerve plexuses along these arteries had of course been stripped as far as possible before the ligatures were applied to the internal carotid. The blood pictures, subsequently

mentioned, were also examined in far greater detail than previously. They included a hæmoglobin estimation by the Newcomer plate method, a red cell count, a reticulocyte count, a diameter estimation with the halometer and a microscopic examination of films stained with Leishman's stain.

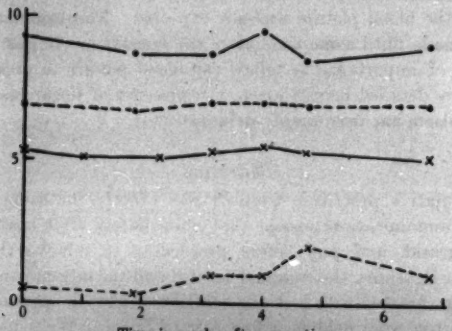
Experimental results

Control experiments

As a control experiment, removal of the right vagus nerve in the neck was performed under ether anæsthesia, and the blood picture followed.

Five animals were used. The blood picture was examined before operation and subsequently at regular intervals. The hæmoglobin content, the red cell count and diameter alone were estimated. These values were found to be constant within physiological variations. As all these values were normal, a detailed search for megaloblasts or normoblasts was not made. A cursory examination, however, indicated that the stained films seemed normal in every respect.

Operative procedure, therefore, does not markedly affect the blood picture.

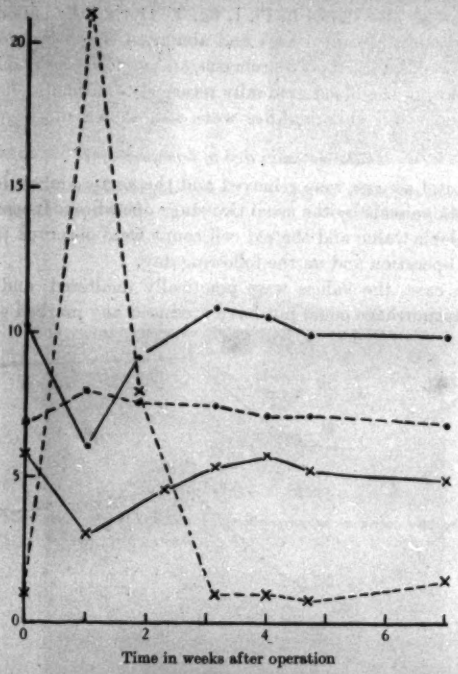


Text-fig. 1. Results obtained with the atypical test animal. ●——● Hæmoglobin (g./100 c.c.). ● - - - ● Diameter (in μ.). ×——× Red cell count (millions/c.mm.). × - - - × Reticulocyte percentage. In subsequent figures, the same symbols are used.

Removal of both sinuses and denervation of aortic area

Four animals were used in all. One of these showed no changes whatsoever after the operation. The various values studied remained remarkably constant and no abnormalities were found in the blood

films. In other words, this animal served the purpose of an almost perfect control. For this reason, the results obtained are shown in graph form in Text-fig. 1. Post-mortem examination of this animal revealed that, owing to a slight anatomical abnormality in the arrangement of the blood vessels, the carotid sinus on the right side had not been removed.



Text-fig. 2. Results obtained after operation, showing the marked variations and high reticulocyte count.

The results obtained with the other three animals amply confirmed those given by the six animals of the previous communication. The blood pictures were studied before the operation and then at regular periods commencing one week later. In each case, the results were essentially similar. The readings in one of these animals are shown in Text-fig. 2.

It will be seen that the initial variations were even greater than those of the previous communication. At one stage, the reticulocyte count reached the remarkable value of 21 p.c., and then eventually returned to normal.

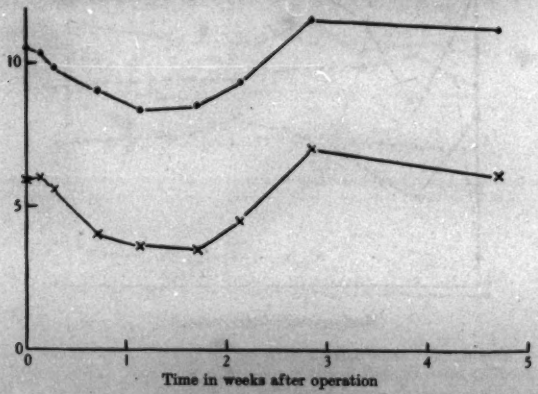
One week after the operation, the film showed both megaloblasts and normoblasts. These are shown in Pl. I, figs. 1 and 2. The marked reticulocytosis is also shown in Pl. I, fig. 3. During the period that the reticulocyte count remained high and abnormal cells were present, the red cells showed a marked polychromasia, anisocytosis and poikilocytosis. As time went on, the blood gradually returned to normal.

The various cell abnormalities were also shown in the other two animals.

Is the anæmia due to hæmorrhage?

The carotid sinuses were removed and the aortic arches denervated in each of six animals by the usual two-stage operation. In each animal, the hæmoglobin value and the red cell count were observed just before the second operation and on the following day.

In each case, the values were practically unaltered, and thus the operative hæmorrhage could not have produced any marked effect.



Text-fig. 3. Results obtained after operation, showing the slowly developing anæmia.

Unfortunately, two of the animals died later from operative shock. Therefore, the hæmoglobin values and cell counts could be subsequently followed in only four of the animals. This was done at short intervals, which at first did not exceed 3 days.

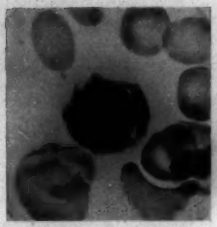


Fig. 1. Blood film showing a megaloblast. ×1500.

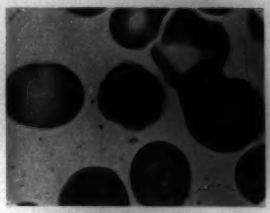


Fig. 2. Blood film showing a normoblast. ×1500.

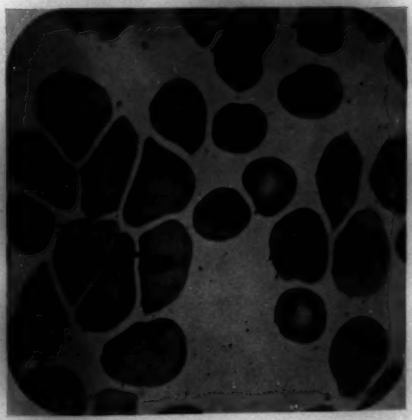


Fig. 3. Blood film showing the marked reticulocytosis. ×1500.

To face p. 78

The results, in one case, are shown in Text-fig. 3. It will be seen that the anæmia was produced relatively slowly, taking 1 week to 10 days to reach its maximum intensity. It could not, therefore, have been produced by operative hæmorrhage. Further, all blood lost at each of the six operations was carefully dabbed up on cotton-wool swabs and their total iron content estimated. This indicated that the average blood loss for each operation was in the neighbourhood of 3 c.c., and therefore obviously much too small to produce an anæmia.

Discussion

It has, therefore, now been shown in each of thirteen animals that removal of the carotid sinuses and denervation of the aortic arch markedly affects the red blood cell picture. The question now arises as to the exact mechanism whereby these alterations are produced. There are a number of possibilities to be discussed.

In the first place it seems possible that removal of the internal carotids might produce ischæmia of a part of the brain concerned with blood formation. This question may be settled by serial sections of the brains of animals with their carotid sinuses removed. This will be undertaken at the first opportunity.

It is also possible, although perhaps it seems unlikely, that the increased blood pressure, after the removal of the carotid sinuses, might alter the blood picture. Possibly also interference with water exchanges may play a part, altering the blood picture in a manner similar to that shown by Gilman & Goodman [1937] in the case of pituitrin anæmia.

Of all these possibilities, it seems most likely that the carotid sinus, in the normal intact animal, responds to the stimulus of oxygen-lack, and by some reflex means possibly influences the bone-marrow. This is a departure from the view generally held that it is the bone-marrow itself which reacts to oxygen-lack.

A reflex mechanism of reaction would of necessity involve some "centre" in the brain. Some experiments with cats on destruction of areas of the hypothalamus, which are now being performed in this laboratory, are intended to demonstrate this point, which will be fully discussed in a later communication. Indirect evidence for the existence of such a centre has been accidentally obtained using rabbits as experimental animals. In the course of an attempt to hypophysectomize these animals by the method of Firor [1933], it was found that owing to a technical error the pituitary was still intact and that an adjacent area

of brain had been removed. A number of these animals developed a mild hyperchromic macrocytic anæmia.

There is also a certain amount of clinical evidence to suggest the existence of a centre concerned with blood formation. It is well known that in lesions of the pituitary region there is some alteration of the blood picture, e.g. Cushing's basophil pituitary syndrome. This might be due to involvement of a centre in the hypothalamic region.

In the present series of experiments, the abnormal blood cells did not persist after the blood count had returned to normal, as they did in the previous set of six animals. This may be due to the more drastic denervation procedure now adopted.

That the initial anæmia is not due to blood lost at the operation is shown by its gradual onset and by the negligible loss of blood.

Summary

1. Removal of the carotid sinuses and denervation of the aortic arch affect the blood picture, producing a temporary anæmia of the hyperchromic macrocytic variety with a remarkably high reticulocyte count and the presence of primitive blood cells of the red series.

2. In control operations, no such alteration occurred.

3. The significance of these results is discussed, particularly with regard to the existence of a brain centre concerned with red blood cell formation.

In conclusion, I should like to thank Prof. H. E. Roaf for much helpful criticism, encouragement, and advice.

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J. Physiol. (1938) 93, 81–85 612.117.2:612.647

THE OSMOTIC PRESSURE OF FŒTAL AND MATERNAL SERA IN THE SHEEP

By E. F. McCARTHY

From the Physiological Laboratory, Cambridge

(*Received* 12 *January* 1938)

Cryoscopic measurements on human fœtal and maternal blood have been made by a number of observers. J. Veit [1900] found that the freezing point of human fœtal blood was lower than that of maternal blood. Füth & Wirz [1929] have reviewed the work of Veit and subsequent investigators and failed to confirm Veit's results.

Cryoscopy is suitable only for the detection of comparatively large differences in osmotic pressure. The experiments recorded below deal with the direct measurement of the osmotic pressure of the serum protein. Earlier investigations of serum osmotic pressure have been reviewed by v. Farkas [1935]. In the present work, measurements of the osmotic pressure of buffered solutions of serum proteins have been determined for comparative purposes and no measurements have been made of the pressures exerted by the sera in equilibrium with their ultrafiltrates.

Method

Samples of blood were drawn by carotid arterial puncture from sheep and newborn lambs and left to clot. The electrolyte content of the sera was fixed by dialysis in collodion membranes against Sorensen phosphate buffer mixture containing $\frac{1}{10}$ mol. of potassium dihydrogen phosphate and $\frac{1}{10}$ mol. of disodium hydrogen phosphate per litre of solution. The *p*H value of this mixture is 6·8 at 18° C. and 6·9 at 0° C. The dialysate was tested for protein which was either absent or present in very faint traces. Measurements of the osmotic pressure were made at 0° C. using osmometers as described by Adair [1925]. The same phosphate buffer was used throughout. At the conclusion of the experiments the outer fluids gave negative tests for protein and showed no marked alteration of *p*H by the colorimetric method.

Protein concentrations were calculated by Kjeldahl nitrogen determinations.[1] The ratio of albumin to globulin was considered in these calculations. The total nitrogen of the serum was first estimated and the value for albumin nitrogen was determined as follows: 7 c.c. of saturated magnesium sulphate and 1·2 gm. of the finely powdered salt were added to 1 c.c. of serum, the mixture was well shaken and left to stand overnight in a tightly stoppered 25 c.c. flask. The precipitate was then filtered off and the nitrogen was estimated in an aliquot part of the filtrate. The globulin nitrogen was calculated by subtracting the value obtained from albumin nitrogen from the total nitrogen. The nitrogen content of sheep serum albumin and globulin has not yet been determined. The factors necessary for the calculation of protein concentration from nitrogen estimation were based on the figures for horse serum albumin 15·60 p.c. N and total globulin, 15·13 p.c. [Adair & Robinson, 1930*a*]. The determination of protein concentration by Kjeldahl nitrogen estimation was checked in a number of experiments by the refractometric method as described by Adair & Robinson [1930*b*].

Results

Serum from two maternal sheep and their lambs and of a normal sheep was used in these experiments. The results are shown in Fig. 1 and Table I in which ρ is the osmotic pressure reduced to mm. of Hg at 0° C. and C represents the concentration expressed as grams of protein per 100 c.c. of solution. The points determined for the fœtal sera and the maternal sera approximate to two separate curves. The curve of the normal sheep occupies a slightly higher position than the maternal curves.

Discussion of the significance of the differences observed in the osmotic pressure of the sera must take into account the proportion of albumin and globulin in each, as albumin exerts a higher pressure than globulin. The serum of newborn animals is known to be deficient in globulin [Topley, 1933]. Table II shows the data found in the present experiments.

Whether the observed differences in osmotic pressure are due entirely to variations in the A/G ratio may be inferred from calculation of π_0 as

[1] Some preliminary estimations of total protein concentration in undialyzed sera were also made. The figure obtained for lamb 2 was 4·89 p.c. and for sheep 2, 7·98 p.c. Another lamb serum had a protein concentration of 4·34 p.c. and the corresponding maternal serum contained 6·61 p.c.

defined below. π_0 represents the limiting value of the ratio P/C in the infinitely dilute solution. This value may be determined for a protein solution by extrapolation from curves showing the relationship between the osmotic pressure, ρ, and the protein concentration, C, or by plotting the ratio $\pi = P/C$ or $1/\pi$ as ordinate and C as abscissa [Adair, 1924, 1928].

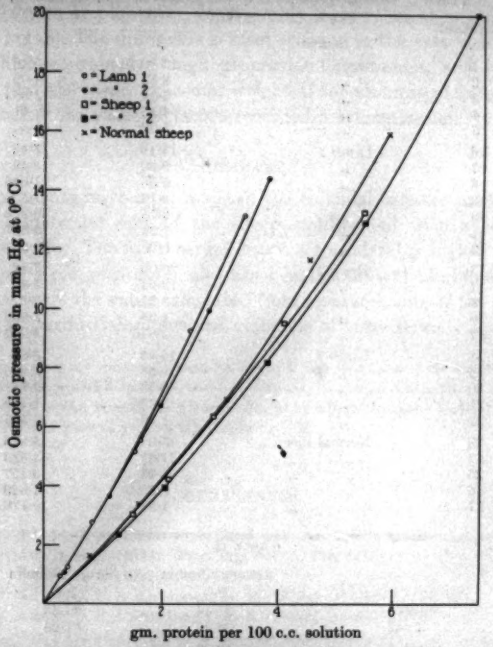

Fig. 1. Description in text.

The value thus obtained for π_0 observed may now be compared with the theoretical value of π_0 which is calculated as stated below.

Adair & Robinson [1930*a*] have shown that in the limiting case of an infinitely dilute solution of protein the osmotic pressure is determined by the formula $p = RT(10\,C_a)/M_a + RT(10\,C_g)/M_g$.

C_a and C_g = g. of albumin and globulin per 100 c.c. of diluted serum. M_a and M_g denote the molecular weights of serum albumin and serum globulin.

The molecular weights of the albumins and globulins of sheep sera are not known, and in the circumstances the values for horse serum proteins, $M_a = 72{,}000$ and $M_g = 170{,}000$, have been utilized in calculating "theoretical" values for π_0, for comparison with the observed values.

TABLE I. Osmotic pressure of serum protein in mm. Hg at 0° C.

Experiment no.	Source	ρ	C
1	Sheep 1	13·30	5·46
2	"	9·53	4·09
3	"	6·34	2·883
4	"	4·26	2·11
5	"	3·02	1·5
6	Lamb 1	13·19	3·42
7	"	9·30	2·49
8	"	5·56	1·646
9	"	5·17	1·554
10	"	2·73	0·826
11	"	1·24	0·397
12	"	0·95	0·227
13	Sheep 2	20·00	7·42
14	"	12·94	5·49
15	"	8·21	3·8
16	"	3·95	2·052
17	"	2·336	1·263
18	Lamb 2	14·45	3·83
19	"	9·98	2·808
20	"	6·73	1·98
21	"	3·656	1·108
22	"	1·09	0·354
23	Normal sheep	15·97	5·876
24	" "	11·69	4·533
25	" "	6·91	3·127
26	" "	3·35	1·629
27	" "	1·57	0·779

TABLE II

	Serum albumin p.c.	Serum globulin p.c.
Sheep 1	35·44	64·56
" 2	38·75	61·25
Lamb 1	62·03	37·97
" 2	62·07	37·93
Normal sheep	49·07	50·93

TABLE III

Serum	X_a albumin g./g. of total protein	π_0 theoretical	π_0 observed
Sheep 1	0·354	1·47	1·83 ± 0·1
" 2	0·387	1·52	1·65 ± 0·05
Lamb 1	0·620	1·84	3·06 ± 0·1
" 2	0·620	1·84	3·02 ± 0·1
Normal sheep	0·490	1·66	1·80 ± 0·1

The theoretical values are then given by the formula

$$\pi_0 = 2{\cdot}36\, X_a + 1{\cdot}00\,(1 - X_a).$$

The results are shown in Table III.

It will be seen that an increase in the fraction of albumin is correlated with a considerable increase in the value of π_0. The observed values for the sheep serum are, however, considerably higher than those calculated for horse serum. The difference is most striking in the case of the fœtal serum which contained a high proportion of albumin, and it seems probable that the mean molecular weight of the albumin in fœtal serum must be below the value of 72,000 recorded for horse serum.

Summary

Measurements have been made of the colloidal osmotic pressures of fœtal and maternal sera of the sheep equilibrated with a standard phosphate buffer. The fœtal serum exerts a considerably higher osmotic pressure per 1 p.c. of protein, and has a higher content of albumin. The pressure exceeds the value calculated from measurements of the osmotic pressures of purified albumins and globulins of horse sera.

I wish to express my indebtedness to Sir Joseph Barcroft and to Mr and Mrs Adair for their kind advice and help.

The expenses of this investigation were defrayed by a grant from the Medical Research Council for which I wish to express my thanks.

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J. Physiol. (1938) 93, 86–89 612.893:612.816.3

THE TRANSMISSION OF IMPULSES BY GANGLIONIC DIRECT FIBRES

By D. P. C. LLOYD

From the Laboratory of Physiology, Oxford, and the Department of Medical Research, Banting Institute, University of Toronto, Toronto

(*Received* 24 *January* 1938)

Obrador & Odoriz [1936] have described a blocking of impulses, or "inhibition" occurring in the direct fibres of the fifth lumbar ganglion, i.e. the fibres which traverse the ganglion without synaptic relay, as indicated by a decrease in the amplitude of the spike potential of a second volley following a similar volley at intervals from 100 to 600 msec. Since the fifth lumbar ganglion possesses a number of collaterals from these direct fibres, synapsing with cells which send their postganglionic processes into the grey ramus, it was suggested that the "inhibition" might be susceptible of a similar explanation as that advanced by Barron & Matthews [1935] in the interpretation of intermittent conduction in the spinal cord, i.e. that it could be brought about by an electrotonic block at the collateral junction, the source of the electrotonic potential being, presumably, the ganglion cells in anatomical or synaptic relationship with the collateral.

In the inferior mesenteric ganglion, which resembles the fifth lumbar ganglion in possessing direct fibres with collaterals [Lloyd, 1937], no alteration of the spike potential recorded from the direct fibres has been observed, providing the stimulus interval is greater than the relatively refractory period of the fibres. Because of this discrepancy, it was decided to reinvestigate the conditions of transmission by the direct fibres of the fifth lumbar ganglion. In addition the sixth lumbar ganglion has been similarly investigated.

Method

Cats have been used, sixteen in all. Twelve were examined after decerebration and four under the influence of nembutal (40 mg./kg.). After preliminary exposure of the sympathetic chain in the lumbar

region, interganglionic stretches of the chain were isolated for the placing of stimulating and recording electrodes. The ganglion itself was left undisturbed to avoid damage to the blood supply. Break shocks from coreless coils timed by a Lucas pendulum were applied to the chain, the potentials being recorded beyond the ganglion by means of R.C.

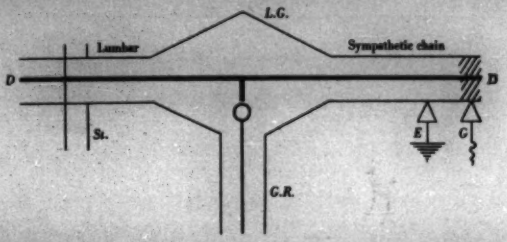

Fig. 1. A diagram of a lumbar ganglion to show the position of stimulating and recording electrodes in relation to the ganglion and direct fibre system traversing the ganglion. Other conduction pathways have been omitted for simplicity. *L.G.* lumbar ganglion; *G.R.* grey ramus; *D*, direct fibre with collateral; *St.* stimulating electrodes; *E* and *G*, earth and grid amplifier leads.

coupled amplifiers and cathode-ray oscillograph. Fig. 1 shows the position of stimulating and recording leads in relation to the ganglion and the direct (*D*) fibre system traversing the ganglion.

Results

As Obrador & Odoriz have shown, a maximal volley applied to the lumbar sympathetic chain gives rise to two distinct spike potentials (*A* and *B* in their terminology) in the chain beyond a ganglion. This is true, not only with the fifth lumbar ganglion, but also with the sixth lumbar ganglion. These spikes have been renamed in the present paper in accordance with the unified system of nomenclature suggested in a previous paper [Lloyd, 1937]. Thus the *A* and *B* spikes as recorded from the chain itself may be called the *D* (direct fibre) spike and the *S* (synaptically relayed) spike respectively (cf. Fig. 2). However, it must be noted that some of the second (*S*) spike is due to direct fibres of slower conduction. It is thus a combined *D* and *S* spike.

In the present experiments submaximal, maximal and supramaximal volleys were applied to the lumbar sympathetic chain in various combinations and with various stimulus intervals. Fig. 2 shows part of the series of observations in each of two experiments, employing volleys

Fig. 2. (A) Action potentials of *D* fibres in the fifth lumbar ganglion evoked by two volleys just maximal for the *D* fibres. Stimulating electrodes on the lumbar sympathetic chain above the ganglion. Recording leads on the chain below the ganglion. (B) A similar experiment, but for the sixth lumbar ganglion. Further explanation in text. Time: 1 d.v. = 10 msec.

just maximal for the *D* spike, the potentials being recorded from the chain beyond the ganglion as indicated in Fig. 1. In Fig. 2A the fifth lumbar ganglion has been used, obs. 1 being the potential from the testing volley alone, obs. 2–6 being the potentials from the two volleys in succession, the intervals varying from 58 to 228 msec. In Fig. 2B a similar experiment on the sixth lumbar ganglion is illustrated, obs. 1 being the control potential from the testing volley alone, while obs. 2–6 are the potentials from the two volleys in succession at intervals from 22 to 233 msec. In both experiments the amplitude of the *D* spike from the testing volley is unaltered throughout the range of stimulus intervals. This has been a constant finding with all combinations of volleys and intervals, provided the interval was sufficiently long to avoid relative refractoriness. It may be concluded that, under the conditions of the present experiments, it is impossible to demonstrate the "inhibition" of direct fibres described by Obrador & Odoriz. Thus the presence of a collateral does not affect the transmission of impulses along the main preganglionic axone traversing a ganglion in any way that can be demonstrated by the present technique. The observations shown in Fig. 2 do not extend over the whole range of intervals at which the blocking effect might occur *ex hypothesi*, but they do include the theoretical optimal region, 50–200 msec., assuming that the rapid appearance and disappearance of electrotonus at a point some distance from the potential source, as described by Bogue & Rosenberg [1934], would obtain in this special case.

SUMMARY

Under the usual conditions of ganglion experiments, it has been impossible to demonstrate "inhibition", or blocking of impulses in direct fibres of the inferior mesenteric ganglia, the fifth or the sixth lumbar ganglia of the cat.

The author wishes to thank Dr J. C. Eccles for suggesting this work, and Dr Donald G. Marquis for his valuable co-operation in the experiments performed in Oxford, and to express his gratitude to the Christopher Welch Trustees for defraying the cost of photographic plates used in Oxford.

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J. Physiol. (1938) 93, 90–103 612.172.2:612.232

THE EFFECT OF ASPHYXIA UPON THE HEART RATE OF RABBITS AT DIFFERENT AGES

By D. J. BAUER[1]

From the Department of Physiology, Cambridge

(*Received* 23 *February* 1938)

It has been shown [Barcroft, 1938] that when the umbilical cord is clamped in fœtal goats towards the end of pregnancy a fall of heart rate is produced, with recovery when the cord is released. This fall is caused partly by the vagi and partly by some other factor, probably depression of the pacemaker by asphyxia, since when the vagi are cut a second clamping of the umbilical cord still causes a fall of heart rate, which, however, appears considerably later. The same is found in the fœtal sheep [Bauer, 1937] where at the end of pregnancy a vagal fall of heart rate occurs within a few seconds of clamping the cord, while in younger fœtuses the fall of heart rate appears after a much longer interval and is non-vagal. Clark [1932], in fœtal cats, found that when the uterus contracted a fall of fœtal heart rate took place, which was unaffected by section of the vagi. Vagal action therefore seems to differ in different animals, and in the experiments described in the present paper the question of vagal participation was investigated in fœtal rabbits, and the observations were extended to include rabbits at different ages after birth in order to see whether any further effects, vagal or otherwise, appear as a result of asphyxia after birth, or whether the final condition is attained *in utero*.

Methods

Method of recording

The object of most of the experiments was to observe the effect of asphyxia upon the heart rate of the animal, so that some method of heart recording is needed which can be used on fœtuses and small animals. Methods involving the recording of blood pressure were tried,

[1] Coutts Trotter student, Michael Foster student.

but were found to be unsatisfactory. Some records were also obtained by opening the thorax and attaching levers to the heart. This method can be applied to fœtal rabbits, but by the time the preparation has been set up for recording the condition of the fœtus has deteriorated very considerably.

For these reasons a record of the heart was obtained by recording the electrocardiogram, a method which is applicable to animals of all sizes. It was led from the limbs to a four stage resistance capacity coupled amplifier, giving a maximum overall amplification of 160,000. As a mechanical record on a smoked drum has many advantages over photographic recording when accuracy of reproduction is not required, an oscillograph of the type introduced by Winton [1936] was used, actuated by a power output stage. The record so obtained was naturally very distorted, but the separate waves of the electrocardiogram were shown as long as the heart rate was lower than 100 per min., and it was always possible to tell when the heart was in block.

Two methods were employed for attaching the electrodes to the limbs. In young rabbits small rectangular plates soldered to the input leads were buried under the skin and secured in position by tape wrapped firmly round the limb. Random movements of the electrodes which would have caused interference in the electrical record were thus avoided. With fœtal rabbits it is essential to obtain the record as soon as possible after opening the uterus, so that the leads were connected to the limbs by crocodile clips for the sake of quickness and a certain amount of interference was tolerated in the record.

Anæsthesia

Urethane was used as the anæsthetic, given subcutaneously or parenterally. The usual dose was 1 g. per kg. in saturated solution, but this was varied according to the degree of anæsthesia required and the age of the animal, since young animals seemed to be more sensitive to its effects. In a number of experiments ether was used, but no difference was observed in the results.

Operative methods

In experiments on fœtuses a low median incision was made in the abdominal wall of the mother and a portion of uterus withdrawn. This was opened sufficiently to allow one fœtus to be taken out. The fœtus was then freed from the amniotic sac and was covered with pads of cotton wool soaked in warm saline, the whole being arranged in such

a way that the umbilical cord was not stretched. The cord was clamped by means of a small bulldog clip, the spring of which had been weakened so that the vessels were occluded without being crushed. Care is necessary in manipulating the cord, since the vessels undergo permanent contraction if they are handled too much. For this reason the same fœtus was seldom used for more than two observations.

Asphyxia was produced in young rabbits by clamping the trachea. In the case of larger rabbits a short cannula with a rubber tube attached was inserted into the trachea and a clamp was placed on the rubber tube. The volume of air available to the animal was thus relatively the same in all cases, being the amount contained in the lungs and that part of the trachea lying below the clamp, so that curves obtained from animals of different sizes are comparable in their time relations.

Results

Fœtal rabbits

When the umbilical cord is clamped the heart rate remains unchanged for a short time and then falls rapidly to less than half the initial rate, as shown in curve *A* of Fig. 1. The same sequence of events still occurs

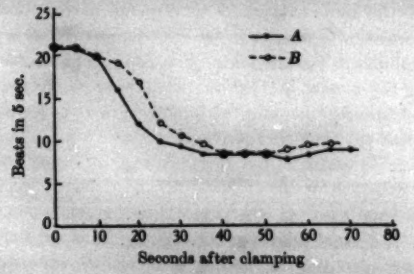

Fig. 1. Asphyxial curve of fœtal rabbit at full term. *A*, normal; *B*, after decapitation.

after the fœtus has been decapitated (curve *B*), the difference between the two curves not being great enough to be significant, so that the fall of heart rate cannot have been brought about by reflex vagal action. Similar results are obtained if the vagi are cut before clamping the cord for the second time, and if the vagi are cut while the fall of heart rate is occurring no rise of rate is produced, showing that vagal inhibition is absent. Asphyxia never produces a vagal reflex *in utero*, since observations made on fœtuses right up to the end of pregnancy show that the fall of heart rate follows the same course after the vagi have been cut.

After birth

When a young rabbit is asphyxiated the heart rate does not follow the same course as in the fœtus. Six main types of asphyxial curve can be distinguished, characteristic of different ages.

(1) The first type is shown in Fig. 2 (curve *A*), appearing at birth and persisting, with some modifications, until the 10th day. It differs from the fœtal type in that the heart rate, which is now somewhat higher than before birth, remains unchanged for the first forty seconds or so of

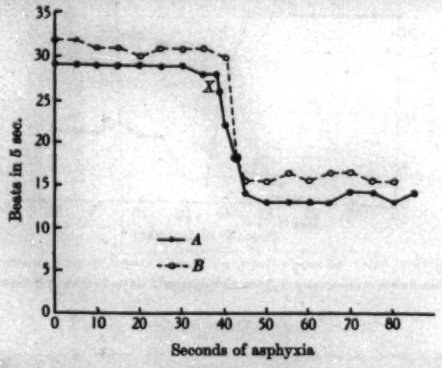

Fig. 2. Asphyxial curve at 21 hours. *A*, normal; *B*, after cutting the vagi.

asphyxia. A fall of rate then occurs which is somewhat more abrupt than the fall seen in the fœtus. When the asphyxia is discontinued the heart rate rises to its former value. If the vagi are now cut a second asphyxiation gives a curve (*B*) which shows no significant difference from curve *A*. A reflex vagal effect is therefore absent.

The difference between this type of curve and the fœtal type probably lies in the change over from placental to pulmonary respiration. To test this point fœtuses were removed from the uterus before the end of pregnancy and were allowed to breathe. Clamping the trachea then produced a curve of the neonatal type. In one experiment in which a fœtus was removed from the uterus 2 days before the end of pregnancy, clamping the trachea 45 min. after the commencement of pulmonary respiration gave the curve shown in Fig. 3.

It will be shown in the discussion that the oxygen saturation of the blood may play an important part in determining the shape of the

asphyxial curve before and after birth. An investigation of this point was therefore carried out, using the Millikan colorimetric method, the results of which are shown in the accompanying table.

Foetuses, 1 or 2 days before term	
Carotid artery (p.c.)	50, 30, 68, 53
Umbilical vein (p.c.)	65, 55, 70
Young rabbits, 20–90 min. after removal from uterus	
Carotid artery (p.c.)	78, 87, 75

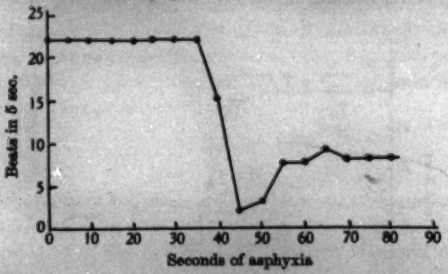

Fig. 3. Asphyxial curve 45 min. after the commencement of pulmonary respiration. The foetus was removed from the uterus 2 days before full term.

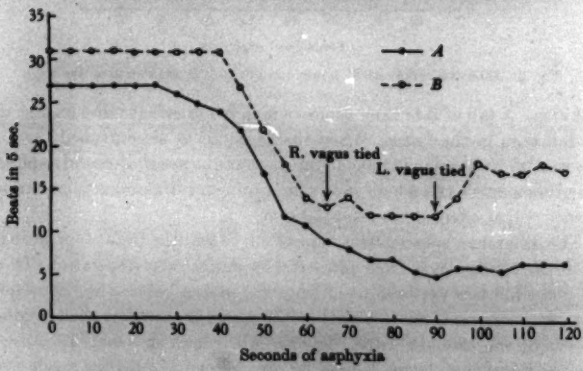

Fig. 4. Asphyxial curve at 4 days. *A*, normal; *B*, vagi tied during asphyxia.

The shape of the asphyxial curve undergoes a modification with increasing age in that the fall of heart rate produced by asphyxia becomes greater, as seen in Fig. 4, from a 4-day-old rabbit. In a number of experi-

ments loose ligatures were placed around the vagi and were tied when the asphyxial fall of heart rate had appeared (Fig. 4 *B*). A rise of heart rate appeared after each tying, the extent of which increased with increasing age. A vagal reflex is therefore produced by asphyxia after birth which increases the asphyxial fall of heart rate already present, and increases in strength up to the 10th day.

(2) The second type of curve appears on the 11th day, and is shown in Fig. 5, the curve from a 10-day rabbit being shown at the same time

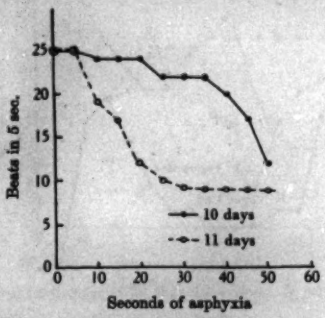

Fig. 5. Asphyxial curves at 10 and 11 days.

for the sake of comparison. A fall of heart rate now appears within the first ten seconds of asphyxia. If the asphyxiation is repeated after cutting the vagi a curve of the form shown in Fig. 2 appears. The fall of heart rate is thus brought about by the vagi and probably represents the final state of development of the vagal effect which gradually appears during the first 10 days. If the vagi are ligated just after the fall of rate has occurred a rise immediately occurs which brings the heart rate back to its initial value.

From a consideration of the results so far presented the vagal effect might develop in one of two ways: either the vagi have been capable of slowing the heart from an early age, and are not reflexly invoked to any great extent until the 11th day, or else the conditions necessary in asphyxia for eliciting a vagal reflex have always been present but have been unable to do so owing to the fact that the action of the vagi on the heart does not appear in its full form until the 11th day. This question was investigated by stimulating the vagi in rabbits of different ages by

means of an induction coil. The findings may be summarized as follows:

(*a*) A stimulus which is just adequate to slow the heart in rabbits older than 10 days is inoperative in animals of earlier ages. A quantitative investigation showed that the threshold strength falls during the days just after birth and reaches a minimum at 11 days.

(*b*) If the vagi are stimulated subliminally during asphyxia, slowing of the heart is produced. In all the experiments upon which these results are based the vagi had been ligated and cut before stimulation.

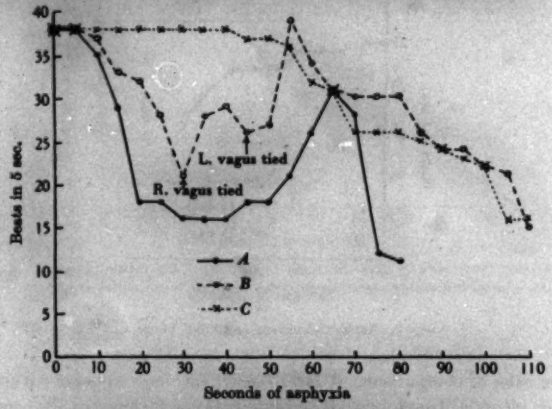

Fig. 6. Asphyxial curve at 20 days. *A*, normal; *B*, vagi tied during asphyxia; *C*, vagi tied previous to asphyxia.

(3) The third type of curve first appears at 12 days and reaches its full development at 20 days. It is characterized by a marked rise of heart rate occurring after about 50 sec. of asphyxia (see Fig. 6, curve *A*). Ligature of the vagi (curve *B*) is followed in each case by a rise of rate and the latter part of the curve now lies at a much higher level than when the vagi were intact. This shows that the vagi are concerned in the second fall of rate which takes place after the rise has appeared, so that the rise cannot be a result of vagal escape. Curve *C*, with the vagi cut before the commencement of asphyxia, differs from the non-vagal curve already seen (see Fig. 2), in that the final part (from 50 sec. on) lies at a higher level. It therefore seems that the rise is present here as well, and that it is a positive effect, independent of the vagi.

It was considered likely that the rise of heart rate was a sympathetic effect, and this view was confirmed by its absence after injecting ergotoxin. As ergotoxin has a twofold effect upon the sympathetic system, first stimulating and then paralysing its motor activities, a large dose was always given in order to make sure of getting the second effect. The results of a typical experiment on a 20-day rabbit are shown in Fig. 7. The first asphyxiation gave curve *A*, of the type already seen.

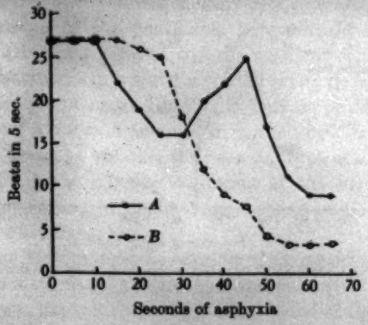

Fig. 7. Asphyxial curve at 20 days. *A*, normal; *B*, after ergotoxin.

After recovery, 5 mg. of ergotoxin ethanesulphonate (Burroughs-Wellcome) dissolved in 0·3 c.c. saline were injected intravenously. Five minutes later, when respiration was becoming irregular, the animal was asphyxiated for the second time (curve *B*), when it was found that the rise of rate had disappeared.

(4) The next type appears from the 30th to the 36th day, and is shown in Fig. 8, curve *A*. The first part of the curve is the same as in type 3, but the rise of rate is cut short soon after its appearance and is replaced by a profound inhibition which is usually great enough to cause complete arrest of the heart. After a short and variable interval the heart rate begins to rise once more but never to any great extent. Ligature of the vagi at the appropriate times shows that both falls of heart rate are vagal (curve *B*), the second fall not being so abrupt, presumably because only one vagus remained to bring it about. Cardiac arrest does not always occur, the characteristic feature of this type of curve being the abolition of the rise of rate and its replacement by a profound inhibition. The abolition of the rise is progressive, being incomplete at 30 days, gradually developing until at 36 days no rise is seen.

(5) After 36 days a further type appears, shown in Fig. 9. The rise of rate has disappeared and the inhibition which followed it now occurs

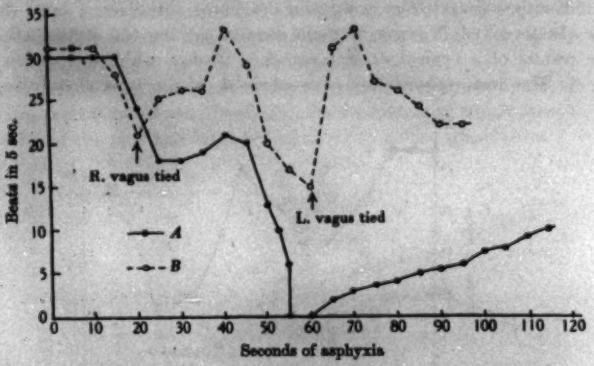

Fig. 8. Asphyxial curve at 32 days. *A*, normal; *B*, vagi tied during asphyxia.

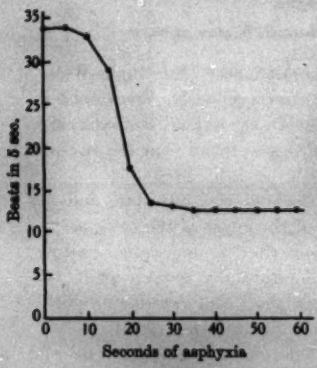

Fig. 9.

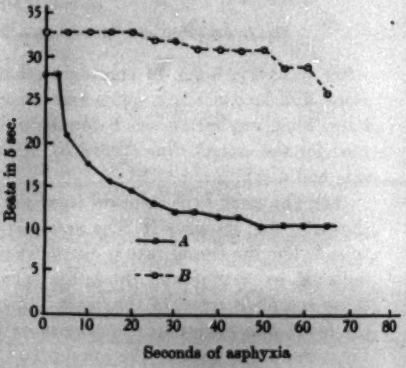

Fig. 10.

Fig. 9. Asphyxial curve at 42 days.

Fig. 10. Asphyxial curve of adult rabbit. *A*, normal; *B*, after cutting the vagi.

earlier in asphyxia, so that the initial fall of rate is greater. This type of curve gradually merges into the final adult type.

(6) This adult type is distinguished from the preceding only in the abruptness with which the initial fall takes place (Fig. 10, curve *A*). When the vagi are cut the asphyxial curve takes on a form which is essentially the same as that already seen (Fig. 6, curve *C*).

Discussion

Since vagal effects are absent in asphyxia in the fœtus and neonatal rabbit it is probable that the fall of heart rate produced in each results from the depression of the pacemaker by asphyxia or its products. The difference between the fœtal and the neonatal curve is such as to suggest that the fœtus is in a state of partial asphyxiation and is in fact working at some such point as *X* in Fig. 2. This supposition is supported by the fact that if a fœtus is removed from the uterus before the end of full term and allowed to breathe, the neonatal type of curve appears on asphyxiation. That the oxygen saturation of the blood does in fact increase after the onset of pulmonary respiration is shown by the determinations quoted.

The following factors must be considered in regard to the difference observed between the fœtal and the neonatal curves:

(1) The oxygen saturation of the arterial blood of the fœtus *in utero* may actually be higher than that found under experimental conditions. Exposure of the uterus may cause its blood vessels to constrict, and opening it produces a certain amount of unavoidable hæmorrhage, so that the maternal blood supply to the placenta is liable to be impaired. The exposure, and particularly manipulation, of the umbilical cord tends to make its vessels close up, so that the blood supply from placenta to fœtus is restricted. For these reasons the supply of oxygen to the fœtus under experimental conditions may be lower than normal, so that the fœtus is in a partially asphyxiated state, while *in utero* its condition may approximate much more to that of the rabbit after birth.

(2) According to Mossman [1926] the fœtal and maternal blood streams run in opposite directions in the placenta of the rabbit. The fœtal blood which leaves the placenta has therefore had an opportunity of coming into equilibrium with the maternal arterial blood which has just entered, so that the oxygen saturation of the fœtal arterial blood may be almost as high as that of the maternal blood.

(3) The two previous points suggest that the fœtus may be well supplied with oxygen as far as the blood in the umbilical vein is concerned, but the point to be considered is the composition of the blood going to the pacemaker. The oxygenated blood from the umbilical vein

suffers a dilution with unsaturated blood from the lower limbs in the inferior vena cava, so for this reason alone the saturation of the blood going to the pacemaker will be less than that of the blood coming from the placenta. Whether any further dilution occurs depends upon whether the Sabatier theory is correct. This states that the blood coming from the inferior vena cava is shunted by the Eustachian valve through the foramen ovale and so into the left ventricle, while the blood from the superior venæ cavæ is directed to the tricuspid orifice by the intervenous tubercle. In this way the two blood streams would cross in the right auricle without mixing, so that the aorta would receive blood of arterial quality from the inferior vena cava without admixture with unsaturated blood from the superior venæ cavæ [Kellogg, 1930]. This theory is based on anatomical appearances only, and was tested experimentally by Pohlmann [1907] by injecting suspensions of starch grains into the superior and inferior venæ cavæ and estimating the amounts of starch in the two ventricles. It was found that the two blood streams mixed completely in the right auricle. Kellogg [1930] repeated this work in pig and dog fœtuses, using the gas contents of the blood as an index, and he found that the oxygen and carbon dioxide percentages of the blood in the two ventricles were very nearly equal, suggesting that almost complete mixing must have occurred. The oxygen saturations of his samples, however, were always very low, so that the blood must have undergone a considerable autoreduction before analysis. Barcroft [1936] determined the oxygen percentages in the carotid artery, aorta and inferior vena cava in fœtal sheep, and found that crossing of the streams did occur, but was by no means complete.

If the two streams from the venæ cavæ cross in the right auricle without mixing, the blood which enters the aorta and is supplied to the pacemaker *via* the coronary vessels will be of the same saturation as the blood in the inferior vena cava. If, on the other hand, mixing does take place, the blood coming from the inferior vena cava will suffer a further dilution. Since the carotid arteries come off from the aorta before the ductus arteriosus, measurement of oxygen saturation of the blood in them will show whether mixing has taken place or not. The figures quoted in the experimental section show that on an average the oxygen saturation is considerably lower in the carotid arteries than in the umbilical vein, so that it seems that crossing of the streams is incomplete, the blood going to the coronaries suffering a further lowering of oxygen saturation. This is confirmed by the figures given for young rabbits, the carotid oxygen saturations being in all cases greater than those of the fœtuses.

It seems reasonable therefore to suppose that an increase in saturation of the blood going to the pacemaker takes place after birth.

(4) This increased saturation is sufficient to explain why the heart rate is higher after birth, but the fact that the fall of heart rate in asphyxia takes place after a much longer interval in the neonatal curve requires that the animal shall have a greater total amount of oxygen available at the commencement of asphyxia. When the umbilical cord is clamped, the fœtus is cut off from the blood in the fœtal placental vessels and only has at its disposal the oxygen of the blood actually within it. At birth the blood within the placenta passes into the neonatal animal, which therefore contains more blood than did the fœtus, so that its total oxygen content is higher. This was shown by Cohnstein & Zuntz [1884] in neonatal rabbits, and by Barcroft & Gotsev [1937] in lambs. An extra amount of oxygen is also available to the neonatal animal from the air which is present in the lungs at the beginning of asphyxia.

From a consideration of the above four points it seems reasonable to suppose that sufficient difference exists between the oxygen content of the fœtus and the neonatal animal to account fully for the observed difference in the asphyxial curves. It should perhaps be mentioned here that it is not considered that the pacemaker is depressed by anoxæmia. It is more likely that accumulation of carbon dioxide is the responsible factor, the oxygen saturation figures being quoted merely as providing a convenient index of the extent to which asphyxial products are present.

Analysis of the vagal effects appearing in asphyxia shows that two distinct manifestations of inhibition are developed. The first manifestation of inhibition appears very early in extreme asphyxia as a reduction of heart rate and it becomes more marked with increasing age until a great slowing is produced right at the beginning of asphyxia. Stimulation experiments show that a stimulus which is above threshold for an 11-day rabbit fails to slow the heart at earlier ages under ordinary conditions but will do so if the animal is asphyxiated. The stimulation threshold also falls with increasing age and reaches a minimum at 11 days just as the vagal effect in asphyxia increases and reaches a maximum at 11 days. It therefore seems probable that the conditions necessary for evoking the vagal effect in asphyxia have always been present and that the gradual appearance of the effect is due to the gradual development of vagal action.

The second manifestation of inhibition becomes evident at the age of 30 days or so as a curtailment of the sympathetic rise of rate which is

soon abolished completely. This inhibition then appears at an earlier stage in asphyxia and blends with the first, so that the fall of heart rate becomes more profound. The factors which are responsible for producing these inhibitions have been investigated and will be described in a further paper.

Summary

1. When the umbilical cord of a fœtal rabbit is clamped a fall of heart rate is produced which is non-vagal.

2. When a young rabbit is asphyxiated some hours after birth a fall of heart rate occurs which is also non-vagal, but which appears only after 40–50 sec.

3. The delay in the appearance of the fall of rate is probably a result of the difference in oxygen content of the fœtus and neonatal animal.

4. Vagal inhibition first appears as an increased reduction of the heart rate in extreme asphyxia. By the 11th day it is so far developed as to be able to cause a profound fall of heart rate a few seconds after the beginning of asphyxia.

5. Vagal activity as tested by direct stimulation develops in the same way as in asphyxia, reaching a maximum at 11 days.

6. From the age of 11 days a rise of heart rate appears after 40–60 sec. of asphyxia, attaining a maximum at 20 days. It is not caused by vagal escape, since it is followed by a fall of rate in which the vagi participate.

7. This rise is abolished by ergotoxin, and is presumably due to a sympathetic effect.

8. From the 30th to the 36th day the rise of rate is cut short and followed by an intensified inhibition which may be sufficient to arrest the heart completely. This inhibition is vagal.

9. From the 36th day onward the rise of rate is completely abolished by inhibition, so that the effect of sympathetic excitation on the heart rate produced by asphyxia is masked.

10. The changes seen in the asphyxial curve are thus caused by two distinct vagal effects, the first appearing in advanced asphyxia and rising to a maximum at 11 days, the second appearing at 30 days, occurring earlier in asphyxia and blending with the first affect.

Thanks are due to Sir Joseph Barcroft for advice, and to Dr Millikan for assistance in determining oxygen saturations.

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SEGMENTAL DISTRIBUTION OF CERTAIN VISCERAL AFFERENT NEURONES OF THE PUPILLO-DILATOR REFLEX IN THE CAT

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(*Received* 17 *March* 1938)

In a previous paper [Irving *et al.* 1937] it was shown that impulses are conducted to the central nervous system from the stomach and small intestine by visceral afferent fibres contained in the right and left splanchnic and the right and left vagus nerves.

Little information is available of the segmental distribution of the visceral afferent neurones in the splanchnic nerves. Among the earliest references are those of Head [1893] and Thorburn [1893], who noted the skin areas to which pain from a particular viscus was referred, and from the nerve supply of these skin areas they deduced the segmental supply. Similar methods have been used by more recent workers; in particular by Gaza [1924], Miller & Simpson [1924], and Bloomfield & Polland [1931].

Kappis [1913] working with dogs used pain reactions as indices of afferent impulses, and found the whole abdominal cavity insensitive to any kind of stimulus after transection of the spinal cord above the level of the 6th thoracic roots. A similar method has been used by Fröhlich & Meyer [1922], who found that in dogs pain fibres from the small intestine did not enter the spinal cord above the 4th thoracic segment. In 1930 Lebedenko & Brjussowa observed changes in pulse rate, blood pressure and respiration on stimulating the gut, and were able to show that afferent neurones from any one part entered a large number of segments of the spinal cord.

By the use of histological methods Ranson & Billingsley [1918] showed that in the cat afferent fibres are most numerous from the 7th to the 10th thoracic white rami. Lebedenko & Brjussowa [1930]

removed various viscera and allowed nerve degeneration to take place. They claim to have observed histological changes occurring in the cells of the sympathetic ganglia. Their results by this method suggest a much narrower distribution than was indicated by their other observations.

A table is given (Table I) summarizing the results of previous investigators. It will be seen that the widest segmental distribution has been described by Lebedenko & Brjussowa.

TABLE I. Segmental distribution as described by previous workers

	Cardia	Stomach	Pylorus	Duodenum	Small intestine	Cæcum
Bloomfield & Polland (man)	T 7–T 9	—	—	—	—	—
Fröhlich & Meyer (dogs)	—	—	—	—	Not above T 4	—
Gaza (man)	T 6–T 7	T 6–T 9	—	—	T 9–T 10	—
Head (man)	T 6–T 7	T 6–T 9.	T 9	—	T 9–T 12	T 9–T 12
Kappis (dogs)	—	T 6–T 9	—	—	T 7–T 13	—
Lebedenko & Brjussowa (dogs):						
By stimulation	—	T 5–L 4	—	T 5–T 12	—	—
Histologically	—	T 7–T 9	—	T 8–T 11	T 6–T 11	T 11–T 12

We report in this paper the result of our studies on the segmental distribution of the visceral afferent neurones of the pupillo-dilator reflex found in the splanchnic nerves.

METHODS

Our observations have been made on cats. Anæsthesia was induced with ether and maintained with intravenous chloralose (0·08 g./kg.). Dilatation of the pupil was the reflex response generally used as an index of afferent fibres, but in some experiments reflex changes of blood pressure resulting from distension of the stomach and intestine were also recorded. The experimental technique was similar to that previously described [Irving & McSwiney, 1935; Irving *et al.* 1937]. Large balloons previously calibrated were used to distend the body and fundus of the stomach. Smaller balloons were inserted into the small intestine and pylorus. A calibration curve of the pyloric and intestinal balloons is given in Fig. 1. Using the calibration curve it was possible to ascertain approximately the pressure exerted by the wall of the viscus. Total pressures recorded and cubic centimetres of air used in the experiments are given in the tables. The vagus and cervical sympathetic nerves were cut in the neck in all experiments, and in some instances the phrenic nerves were cut in the thorax to abolish the afferent pathway from the

diaphragm. Transection of the spinal cord, spinal root section or section of the sympathetic chains in the thorax was carried out if required. Details of procedure are described later in this paper.

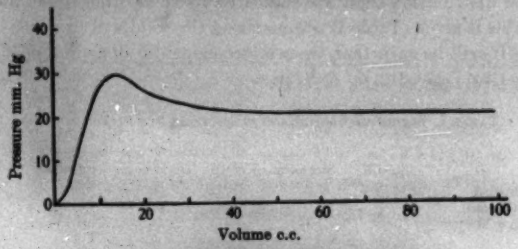

Fig. 1. Calibration curve of typical balloon.

Experimental results

The experiments were divided into three main groups:

(*a*) Experiments to find the upper limit of entry of afferent neurones into the spinal cord from different parts of the stomach and small intestine.

(*b*) Experiments to find the lowest level of entry into the cord.

(*c*) Experiments to show that afferent neurones enter the spinal cord between the extremes found in (*a*) and (*b*).

(*a*) *Upper limit of entry of afferent neurones into the spinal cord*

Bain *et al.* [1935] have shown that afferent fibres in the splanchnic nerves enter the spinal cord *via* the dorsal roots from the 3rd thoracic to the 1st lumbar segment inclusive. In those experiments no evidence was obtained that afferent neurones in the splanchnic nerves from the abdominal viscera enter the spinal cord above the 3rd thoracic segment.

In the first series of experiments we attempted to find the upper level of entry of afferent neurones into the spinal cord from different regions of the stomach and small intestine. Balloons were inserted, the vagus nerves were cut in the neck, and the spinal cord transected in the middle or lower thoracic region. The balloons were inflated and the pressures required to produce a dilatation of the pupil (the threshold pressures) were observed. The lowest conducting spinal root on each side was then cut and the cat was allowed to rest until the pupil constricted, when new threshold pressures were established. Spinal roots were cut until the upper conducting roots were identified. When the cord had been cut

between T 6 and T 7 and three or four afferent roots were conducting impulses into the cord, the section of the 6th dorsal roots was not found to alter appreciably the threshold pressures. As further conducting roots were cut the effect on the threshold became more marked until, finally, section of one pair of roots, either raised the threshold from below 100 to over 200 mm. Hg, or abolished the pupil response completely. We therefore concluded that this last pair of roots was the upper limit of entry of afferent fibres for that particular part of the gut (Fig. 2).

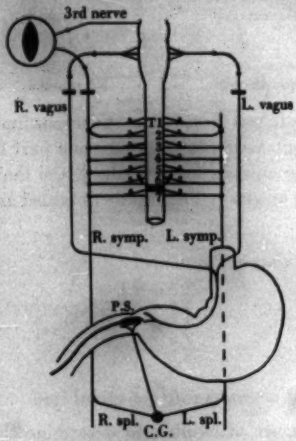

Fig. 2 A.

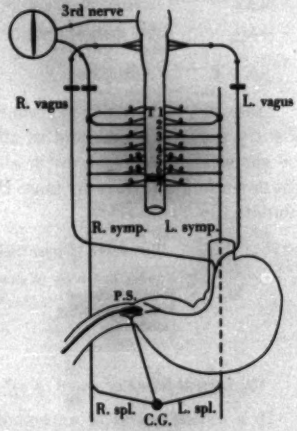

Fig. 2 B.

Fig. 2. A. Cord transected between 6th and 7th thoracic segments. Right and left vagus, and right and left cervical sympathetic nerves cut in the neck. 6th thoracic dorsal roots cut on both sides. Dilatation of the pupil on distension of the pyloric sphincter. B. Dilatation abolished after section of the right and left 5th thoracic dorsal roots.

The consistency of the results obtained from a large number of similar experiments supports this view, and we consider that few, if any, afferent neurones enter the spinal cord above this level from the particular area of the gut.

It has been shown previously [Irving *et al.* 1937] that dilatation of the pupil obtained on distension of the gut with pressures of less than 100 mm. Hg is due to stimulation of nerve endings in the muscle wall. Reflex responses obtained with pressures above 100 mm. Hg are possibly due to traction on nerve trunks. In these experiments, therefore, we

have only used pressures up to 100 mm. Hg. The results of a typical experiment are given in Table II:

TABLE II. After section of spinal cord below T 6, section of 6th thoracic roots and section of vagus in neck

	Fundus Threshold stimulus	Pyloric sphincter Threshold stimulus	Jejunum Threshold stimulus
3.20 p.m.	30 mm. Hg (9 c.c.)	100 mm. Hg (11 c.c.)	No response
3.40	5th thoracic roots cut both sides	—	—
4.30	40 mm. Hg (9 c.c.)	No response	No response
4.35	4th thoracic roots cut both sides	—	—
5.00	No response	No response	No response

From this and similar experiments it was found that the upper limit of entry into the spinal cord of afferent neurones from any one part of the gut was almost constant in all the animals used. Indeed, the limit was never found to vary by more than one root. The results obtained are tabulated in Table III:

TABLE III. Upper limit of entry into spinal cord

Fundus and body of stomach	T 3 or T 4
Pyloric antrum	T 5
Pyloric sphincter	T 5
Duodenum	T 6
Jejunum	T 7

(*b*) *Lowest levels of entry of afferent neurones into the spinal cord*

In a second series of experiments, we attempted to determine the lowest levels of entry of afferent fibres from different regions of the stomach and small intestine. Balloons were inserted into the gut and the vagus nerves cut in the neck. In our preliminary experiments the sympathetic chains were cut on both sides in the thorax above the level of the 9th thoracic rami. After the threshold pressures for the different balloons had been obtained the 9th roots on each side were cut and a new threshold obtained. The process of root section was continued caudally. It was found that when the 12th thoracic roots were the highest roots conducting impulses, a positive pupil response was still obtained from all parts of the viscera under examination. We therefore concluded that afferent neurones from the stomach and small intestine entered the cord below the 11th dorsal roots.

In subsequent experiments the sympathetic chains were cut in the thorax between the 11th and 12th thoracic rami and the threshold

pressure obtained. The thorax was then reopened, the sympathetic chains were cut between the 12th and 13th thoracic rami, and the 12th thoracic segments of the chains removed; new thresholds were then established. Successive segments of the sympathetic chains were removed caudally until the pupil response was abolished or a marked increase in the threshold pressure was required to elicit the reflex.

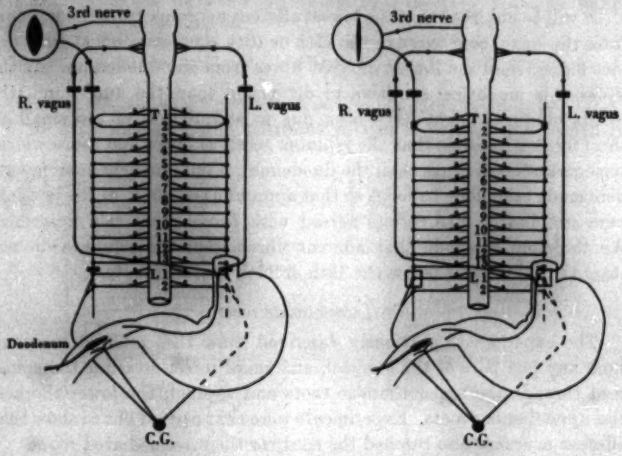

Fig. 3A. Fig. 3B.

Fig. 3. A. Right and left vagus and right and left cervical sympathetic nerves cut in the neck. Right and left sympathetic chains cut between T 13 and L 1. Dilatation of the pupil on distension of the duodenum. B. Dilatation abolished after removal of segment of right and left sympathetic chains at level of 1st lumbar roots.

TABLE IV. After section of the sympathetic chains between T 13 and L 1

	Pyloric sphincter Threshold stimulus	Duodenum Threshold stimulus
3.45 p.m.	75 mm. Hg (6 c.c.)	95 mm. Hg (3 c.c.)
4.00	Sympathetic chains cut between L 1 and L 2	—
4.45	95 mm. Hg (9 c.c.)	No response

In a certain number of experiments no response was obtained on distension of the pyloric sphincter after section of the sympathetic

chains between L 1 and L 2. From this and similar experiments the lower limits given in Table V were obtained.

TABLE V. Lowest dorsal roots conducting

Fundus and body of stomach	T 13
Pyloric antrum	T 13
Pyloric sphincter	L 1 or L 2
Duodenum	L 1
Jejunum	T 12 or T 13

It will be observed that the lowest afferent neurones from the jejunum enter the spinal cord through the 12th or 13th thoracic roots at least one root higher than the lowest afferent fibres from the duodenum. As the jejunum is much less sensitive to distension than the duodenum, the results obtained might have been due to stimulation of too small an area; for it is possible that the jejunum receives fewer and more widely dispersed afferent fibres than the duodenum. Consequently experiments were made using long balloons so that approximately 3 in. of the jejunum were stimulated. The results agreed with those previously described. We therefore conclude that afferent fibres from the jejunum do not enter the spinal cord below the 12th or 13th thoracic roots.

(*c*) *Intermediate roots*

The experiments previously described show that afferent neurones from any one part of the stomach and small intestine enter the spinal cord through the upper thoracic roots and through the lower thoracic and upper lumbar roots. Experiments were next carried out to show that afferent neurones also reached the cord *via* the intermediated roots.

A separate experiment was performed for each pair of roots from the 6th thoracic to the 12th thoracic segment inclusive. For example, when it was intended to leave the 7th thoracic roots as the sole afferent conducting pathway the preliminary procedure was the same as that used in the earlier experiments. In a cat anæsthetized with chloralose, balloons were inserted into the pyloric antrum, the duodenum and the jejunum and the vagus and cervical sympathetic nerves were cut in the neck. The spinal cord was transected at the level of the 8th thoracic segment to exclude impulses reaching the mid-brain *via* roots below T 7. The sympathetic chains were cut in the thorax between the 5th and 6th thoracic rami. By this procedure the 6th and 7th thoracic roots were the only pathways conveying impulses from the gut to the spinal cord.

After the threshold pressures required to elicit dilatation of the pupil were obtained, the sympathetic chains were cut between the 6th and 7th thoracic rami, leaving only the 7th thoracic roots conducting.

Distension of the gut still elicited dilatation. Section of the 7th thoracic dorsal roots, however, abolished the pupil response. The threshold pressures obtained in this experiment are given in Table VI.

TABLE VI. With 6th and 7th thoracic roots conducting

	Pyloric antrum Threshold stimulus	Duodenum Threshold stimulus	Jejunum Threshold stimulus
4.15 p.m.	60 mm. Hg (5 c.c.)	70 mm. Hg (4 c.c.)	70 mm. Hg (7 c.c.)
4.25	After section of 6th thoracic roots	—	—
5.15	100 mm. Hg (7 c.c.)	95 mm. Hg (6 c.c.)	80 mm. Hg (8 c.c.)

Similar experiments were carried out for the other thoracic roots from T 6 to T 12 inclusive, the positions of the balloons being varied in

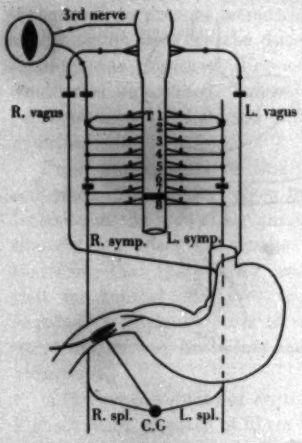

Fig. 4 A.

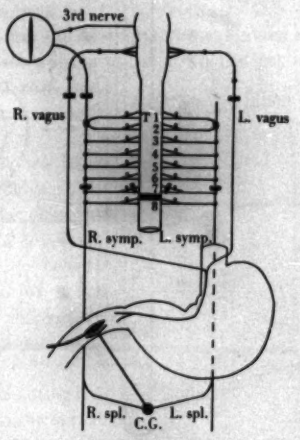

Fig. 4 B.

Fig. 4. A. Cord transected between 7th and 8th thoracic segments. Right and left vagus and right and left cervical sympathetic nerves cut in the neck. Right and left sympathetic chains cut between 6th and 7th thoracic roots. Dilatation of the pupil on distension of the duodenum. B. Dilatation abolished after section of the right and left 7th thoracic dorsal roots.

different experiments. Without exception it was found that each pair of roots formed part of the afferent pathway. Section of the single conducting pair of roots in each experiment abolished the pupil response to distension of the viscera.

Thus our experiments show that different parts of the viscera receive their afferent innervation from all the dorsal roots between the limits given in Table VII, and shown in Fig. 5.

TABLE VII. Summary of highest and lowest levels of entry into the spinal cord of afferent neurones of the pupillo-dilator reflex from the stomach and small intestine

Area stimulated	Upper limit	Lower limit
Fundus and body of stomach	T 3 or T 4	T 13
Pyloric antrum	T 5	T 13
Pyloric sphincter	T 5	L 1 or L 2
Duodenum	T 6	L 1
Jejunum	T 7	T 12 or T 13

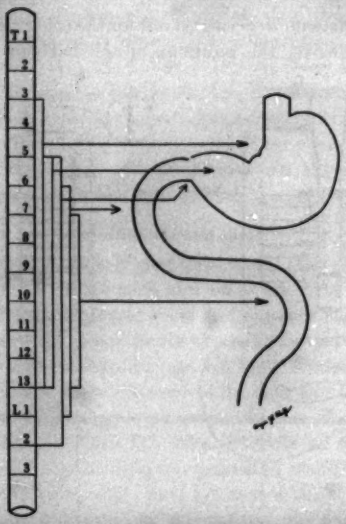

Fig. 5. Diagram to show innervation of different regions of the gastro-intestinal tract.

The experiments already described suggest that there is a wide segmental distribution of afferent neurones of the pupillo-dilator reflex from the stomach or small intestine. Fibres from the stomach and pyloric sphincter apparently enter the spinal cord through no less than ten dorsal roots. Such a wide distribution is contrary to the results of many earlier investigators.

There appeared to be two possible sources of error in our experiments which had not so far been controlled. It was possible that distension of

a balloon in any part of the stomach or small intestine might produce stretching of the muscle in adjoining portions not in contact with the balloon. Or again there might be a spread of nerve impulses through the myenteric plexus to adjacent parts of the gut. Thus distension of the duodenum might produce an effect upon the pyloric sphincter sufficient to stimulate this region, and impulses might be propagated to the spinal cord *via* afferent neurones which had no connexion with the duodenum. Incorrect levels would, therefore, be obtained for the upper and lower limits of entry of afferent neurones into the cord.

Some of the earlier experiments described in this paper on the upper and lower limits were repeated with one variation in experimental procedure. The gut was divided between ligatures above and below a balloon inserted into the duodenum, and purse-string sutures were used, if required, to prevent hæmorrhage from the cut surfaces. In this way all connexion between the duodenum and the adjacent sphincter and jejunum was abolished. Nerve fibres passing up or down the gut wall would also be severed. Subsequent cord and nerve sections were carried out as previously described.

From experiments of this type we have confirmed the results given in Table VII. Isolation of the area of the gut under observation did not effect the results. We conclude, therefore, that there is no reason to believe that mechanical effects or spread of nerve impulses to adjacent parts of the alimentary canal in any way invalidate our previous observations.

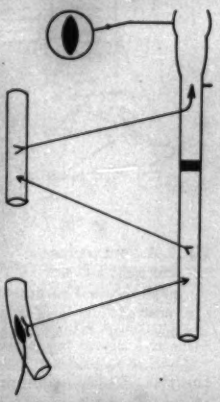

Fig. 6. Diagram to show possible pathway of impulses from isolated segment of gut to upper part of spinal cord.

A further possibility of error concerning the highest point of entry of fibres into the spinal cord was then considered. It seemed possible that in those experiments in which the spinal cord was transected at about the mid-thoracic level, that impulses entering the cord below the transection might interfere with the results. Thus on stimulating, say, the duodenum, an impulse might enter the spinal cord below the level of section and set up a reflex disturbance in some organ, from which a second impulse might enter the cord above the level of section to produce dilatation of the pupil or alteration of blood pressure (Fig. 6).

Experiments were accordingly carried out in which balloons were inserted into the pyloric sphincter and the isolated duodenum. The vagus nerves were cut in the neck, and the spinal cord transected between the 8th and 9th thoracic segments. The threshold pressures required to elicit dilatation of the pupil were then obtained. In addition a blood-pressure record was then taken from the right carotid artery, showing

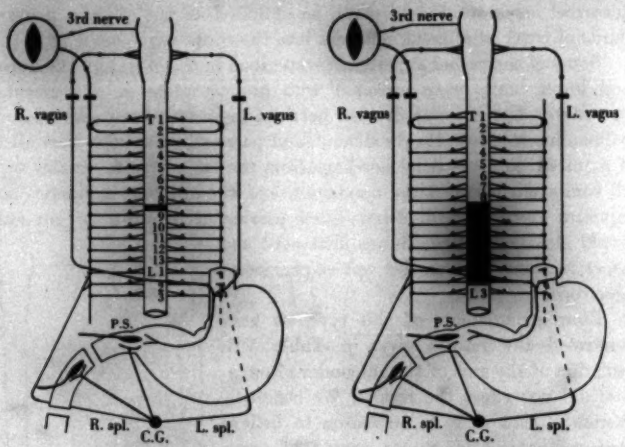

Fig. 7 A. Fig. 7 B.

Fig. 7. A. Cord transected between 8th and 9th thoracic segments. Right and left vagus and right and left cervical sympathetic nerves cut in the neck. Dilatation of the pupil obtained on distension of the pyloric sphincter and the duodenum. B. Cord removed from level of transection to 3rd lumbar segment. Dilatation of pupil on distension of pyloric sphincter and the duodenum.

the rise of blood pressure on distension of the gut. The spinal cord was then exposed from the level of transection to the 3rd lumbar segment, and completely removed between these two limits after section of all the ventral and dorsal roots on both sides (Fig. 7).

The pupil reactions and blood-pressure changes were then observed. The threshold pressures for pupil dilatation are given in Table VIII. It will be seen that removal of this portion of the spinal cord did not produce significant effects. Inflation of either the pyloric or intestinal balloon produced a rise of blood pressure (Fig. 8).

The purpose of taking blood-pressure records in these experiments was to be able to record that the animals were still in a satisfactory condition, despite the considerable vaso-motor disturbance which must necessarily be expected after such a drastic experimental procedure.

Subsequent section of the 7th and 8th thoracic dorsal roots abolished the pupil and blood-pressure responses from the duodenum, but distension of the pyloric sphincter still elicited a dilatation of the pupil (Table VIII), and produced a very slight rise of blood pressure (Fig. 8).

TABLE VIII. Spinal cord transected between the 8th and 9th thoracic segments

Pyloric sphincter Threshold stimulus	Duodenum Threshold stimulus
20 mm. Hg (2 c.c.)	60 mm. Hg (4 c.c.)
After removal of spinal cord below transection to 3rd lumbar segment	
30 mm. Hg (2 c.c.)	70 mm. Hg (4 c.c.)
After section of 7th and 8th dorsal thoracic roots	
100 mm. Hg (4 c.c.)	No response

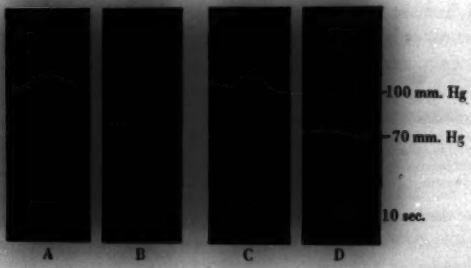

Fig. 8. Tracings to show rise of blood pressure on distension of pyloric sphincter and duodenum after removal of spinal cord from 9th thoracic to 3rd lumbar segment: A. Distension of pyloric sphincter 50 mm. Hg, 2·5 c.c. air. C. Distension of duodenum 75 mm. Hg, 3·0 c.c. air. After section of 7th and 8th thoracic dorsal roots: B. Distension of pyloric sphincter 100 mm. Hg, 4 c.c. air. D. Distension of duodenum 180 mm. Hg, 8 c.c. air.

Thus the highest pair of roots conducting afferent impulses from the duodenum in this experiment was the 7th thoracic, one root lower than that previously obtained. However, we consider that this slight difference lies within the limits of experimental error.

Repetition of these results in subsequent similar experiments show that the conception of a reflex connexion between the upper and lower

halves of the transected cord in the manner suggested is not tenable. The evidence shows that our results are not liable to such source of error.

Discussion

In the chloralosed cat it has been shown that the segmental distribution of the afferent neurones of the pupillo-dilator reflex from the stomach and small intestine is extensive. Previous views of the arrangement of these fibres appear to be based mainly on clinical evidence. If pain fibres from the stomach and intestine have a segmental distribution similar to the pupillo-dilator fibres, then an explanation is afforded for the diffuse character of visceral abdominal pain.

Summary

Dilatation of the pupil and reflex changes of blood pressure in the cat anæsthetized with chloralose have been used as indices of afferent impulses, and evidence has been obtained of the segmental distribution of the afferent neurones of the pupillo-dilator reflex in the right and left splanchnic nerves from the stomach and small intestine.

The highest and lowest levels of entry into the spinal cord of the afferent neurones is shown in Table IX.

Table IX

Area stimulated	Upper limit	Lower limit
Fundus and body of stomach	T 3 or T 4	T 13
Pyloric antrum	T 5	T 13
Pyloric sphincter	T 5	L 1 or L 2
Duodenum	T 6	L 1
Jejunum	T 7	T 12 or T 13

The cost of this investigation has been defrayed in part by a grant from the Government Grant Committee of the Royal Society.

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J. Physiol. (1938) 93, 117–128 612.111:577.159

CARBONIC ANHYDRASE ACTIVITY INSIDE CORPUSCLES. ENZYME-SUBSTRATE ACCESSIBILITY FACTORS

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(*Received* 1 *April* 1938)

Although CO_2 is taken up by serum and converted to bicarbonate only slowly, Dirken & Mook [1931] showed that, when a suspension of corpuscles was suddenly mixed with an equal volume of serum saturated with CO_2, equilibrium as regards CO_2 and bicarbonate was reached in less than a second. They also found that the "shift" of Cl′ into the corpuscles was complete equally rapidly.

Meldrum & Roughton [1933*a*] described the preparation, and studied the properties, kinetics and distribution of the enzyme carbonic anhydrase. They discovered, among other findings, that it occurred in red blood corpuscles; there was no activity in serum. They concluded that "with such large amounts of carbonic anhydrase in the red blood corpuscle it no longer seems difficult to account for the rate of CO_2 evolution in the expired air on the simple bicarbonate theory". In preliminary experiments, with the same technique as had been successful for enzyme solutions, they found, however, that in most cases intact ox and goat corpuscles had less than 0·1 p.c. of the activity of laked corpuscles. They suggested that this low activity of the whole corpuscles "must apparently be a permeability effect of the corpuscle membrane".

Later is was pointed out by Roughton [1935] that, unless H_2CO_3 molecules penetrate into the corpuscles far more quickly than would be expected from their low concentration in the surrounding fluid and from the permeability of the cell membranes to substances of this type, the amount of catalysed dehydration which each corpuscle could accomplish would be limited by its Cl′ content, for only by exchange with Cl′ ions can HCO_3' ions enter the cell. Hence corpuscles in dilute suspensions

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can only perform a small amount of catalysis, and since this occurs in the first few seconds it passes unobserved.

Another important factor of the "accessibility" type, which has not hitherto been pointed out, is the supply—and removal—of H^+ ions inside the corpuscle. Thus in, for example, the dehydration experiments each HCO_3' ion which penetrates into the corpuscle must find a H^+ ion with which to combine before it can be dehydrated: the only quick source of H^+ inside the corpuscles is hæmoglobin, acting as a buffer. Except under conditions approximating to those obtaining *in vivo* the buffer capacity of the hæmoglobin will be inadequate.

For these reasons the manometric technique is unsuitable for assessing velocities comparable with those occurring *in vivo*, but it has proved of value in throwing light on "accessibility" factors, the practical importance of which is brought out by the experiments described in this paper.

Methods

The activity of carbonic anhydrase (or of corpuscles containing it) in accelerating dehydration of carbonic acid was measured in the apparatus described by Meldrum & Roughton [1933*a*] with certain minor changes. Bicarbonate and phosphate buffer are suddenly mixed together in a special reaction vessel and the rate of CO_2 evolution is observed manometrically. The uptake of CO_2 by buffer solutions was done with a modification of the method described by Meldrum & Roughton [1933*b*]. For the experiment illustrated in Fig. 4 the air in the boat was washed out with oxygen, and $\frac{1}{3}$ atm. of oxygen was left in, to ensure that the corpuscles were fully oxygenated throughout the experiment. All the runs in any experiment were done with the same manometer and the curves are plotted directly from the manometer readings against time. In general only the first parts of the curves are shown, although in practice at least one estimation in each experiment was followed to completion in order to determine the total CO_2 evolved or taken up.

Material

Defibrinated blood from the slaughter-house was centrifuged, most of the serum was sucked off and the corpuscles again centrifuged. Any further serum was sucked off and the corpuscles were used without washing unless otherwise stated. For enzyme and other preparations the corpuscles were washed two or three times with saline. Blood drawn from a living animal was ejected from the syringe into a centrifuge tube

containing heparin or a few crystals of potassium oxalate and centrifuged at once. When not stated otherwise pig blood was used.

"Saline" isotonic to 1 p.c. sodium chloride was preferred to simple sodium chloride solution as being more nearly physiological.

Laking accounts for observed activity of corpuscles

In the early experiments when 2 or 3 day old ox corpuscles (washed with 1·5 p.c. sodium chloride) were used a small acceleration of CO_2 output was sometimes observed. That the enzyme liberated by laking caused this catalytic effect was shown by continuing an experiment till no more CO_2 was evolved, centrifuging the contents of the boat and testing the supernatant solution for carbonic anhydrase by adding a portion to the phosphate in another boat experiment. A control for comparison was carried out with a portion of the product from a similar run without corpuscles. Corpuscles from ox, pig, and sheep defibrinated blood and from human oxalated blood have also been tested, but provided the unwashed corpuscles were used as fresh as possible dilute suspensions showed no catalytic activity. Substantially the same results were obtained when the effect of corpuscles on the *uptake* of CO_2 by buffer solutions was studied.

EXPERIMENTS WITH WHOLE CORPUSCLES ILLUSTRATING CERTAIN FACTORS LIMITING THE ACCESSIBILITY OF ENZYME TO SUBSTRATE

Chloride content as limiting factor

An enormous literature now exists on the permeability of the red blood corpuscle membrane. The monograph of Ponder [1934] contains the references until 1934. Controversial points remain, but it is fairly generally agreed that in saline at the *p*H of the blood the membrane is permeable to certain negative ions (HCO_3', Cl', OH', HPO_4'', etc.) and impermeable to positive ions. Davson [1936] has shown that, contrary to earlier beliefs, even in hypertonic solution the impermeability to positive ions persists.

In CO_2 output from blood passing through the lungs HCO_3' enters the corpuscles from the plasma, in which most of the HCO_3' is normally transported, and an equivalent amount of Cl' diffuses out from the corpuscles into the plasma, maintaining electrical neutrality. [The "chloride shift."] The HCO_3' inside the corpuscles then combines with H^+ and dehydrates to CO_2 which diffuses out into the alveolar air *via* the plasma.

In the original protocol for CO_2 output using the two-compartment glass vessel or "boat" the solutions were:

In the right: 2 ml. 0·2*M* $NaHCO_3$ in 0·02*M* NaOH.
In the left: 2 ml. 0·2*M* phosphate buffer *p*H 6·8,
x ml. test solution or corpuscles,
0·5 − *x* ml. water or saline.

The CO_2 output under these conditions was 40 vol. p.c. Henderson [1928] gives the chloride content of cells for man as 21·8 mM. per litre of whole blood. Even if *all* this chloride can "shift" and if the suspension contained the normal 45 p.c. corpuscles it could only account for an output of 47 vol. p.c. But the maximum amount of corpuscles that could be used in these experiments was 2 p.c. The chloride in these last could account at most for only 2·3 vol. p.c.

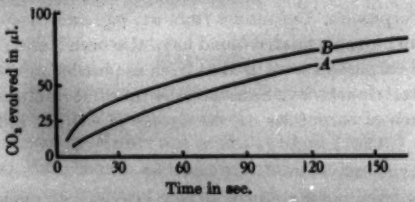

Fig. 1. Effect of intact corpuscles on CO_2 output from phosphate buffer at *p*H 7·4 and 0°. For curve *B*, 0·2 ml. 0·2*M* bicarbonate and 3 ml. 0·2*M* phosphate + 0·5 ml. corpuscles. For curve *A*, saline replaced corpuscles.

The permeability of the corpuscle membrane to glycol and other organic substances is considerably lower than to oxygen [Jacobs, 1933]. If the permeability to H_2CO_3 is similar to that for glycol, with the small concentration gradient of H_2CO_3 obtaining under the conditions of these experiments, inappreciable migration of H_2CO_3 across the membrane may be assumed. As Roughton [1935] pointed out, the Cl′ content of the corpuscles would be the limiting factor. Enzymic acceleration could then only occur at the beginning of the CO_2 output and would easily be missed. Experiments were therefore performed at 0°. The low temperature has two advantages: (*a*) the rates are slower, allowing more accurate readings to be taken, (*b*) any enzymic effect is more marked because, while the uncatalysed dehydration has a temperature coefficient (Q_{10}) of 3·6, that of the catalysed is only 2·1 [Meldrum & Roughton 1933*a*]. An initial acceleration in the rate or "kick" in the curve in presence of corpuscles would then be more easily detected.

Less bicarbonate was used—0·2 ml. instead of 2 ml.—and more phosphate—3 ml. instead of 2 ml.—and the *p*H of the buffer used was 7·4. With these quantities the *p*H remained nearly constant throughout the experiment, and larger quantities of corpuscles could be used—0·5 ml. instead of 0·1 ml. The curves obtained are shown in Fig. 1 in which the initial "kick" is visible. The *total* effect, however, was small and fell off after a few seconds.

Chloride content exhausted by exchange with phosphate

Since the red blood corpuscle membrane is permeable to negative ions, while the corpuscles are standing dispersed in the phosphate during equilibration, Cl' would tend to diffuse out and HPO_4'' to diffuse into them. The concentration of the HPO_4'' outside being high and the volume large, when the Donnan equilibrium is approached the concentration of Cl' remaining inside would be very low. On adding the bicarbonate and commencing the shaking only a negligible quantity of HCO_3' could reach the enzyme by exchanging with the depleted Cl'. Phosphate cannot exchange rapidly with HCO_3' because the former diffuses comparatively slowly across the membrane [Maizels, 1934], and this slower diffusion rate becomes a limiting factor. "Diffusion across the membrane" here includes diffusion through the layers of solution immediately in contact with each side of it.

That chloride does diffuse out from corpuscles while they are suspended in phosphate was shown by estimating their chloride content. Day old corpuscles were used for these estimations, although duplicate estimations were also performed on fresh corpuscles. The corpuscles were in every case washed twice in 6 p.c. glucose and centrifuged for 6 min. at the same speed in similar cups before the estimations were made. The glucose solution removed any chloride in the external fluid wetting the corpuscles and the similar treatment made "packing" and other conditions comparable in each case. For the first estimation corpuscles were obtained as rapidly as possible from fresh pig blood, washed in glucose solution and dealt with immediately. For the second estimation corpuscles were obtained from the same sample of blood by centrifuging 24 hr. later. A further quantity of these day old corpuscles was shaken with phosphate buffer, *p*H 7·3, for 5 min., centrifuged, and divided into two portions. The one was washed in glucose solution and used for the third chloride estimation. The other portion was shaken with excess saline for 5 min., centrifuged, washed in glucose solution and used for the fourth chloride estimation. From each of these four groups

of corpuscles 10 ml. were measured out, laked with water, deproteinized with tungstic acid by the method of Folin & Wu [1919], and made up to 80 ml. Chloride estimations were made in triplicate on 1 ml. samples of this solution by Conway's [1935] method in which chloride is oxidized to chlorine with acid permanganate and distilled into potassium iodide solution in a specially developed micro vessel. The iodine liberated is titrated with thiosulphate. Conway recommends subtracting the titre obtained with a blank experiment on the reagents. But the blank proved negligible when the reagents were freshly made up. The results are summarized in Table I. It will be seen that the chloride content falls slightly

Table I. Chloride content of pig corpuscles

Estim. no.	Age and treatment	mM. Cl′ per litre corpuscles
1	From fresh blood	31·5
2	„ 24 hr. blood	29·0
3	„ „ washed phosphate	3·0
4	„ „ „ then saline	33·0

when blood is kept for a day. This has since been confirmed. The disappearance of most of the chloride on shaking the corpuscles with phosphate is strikingly demonstrated, as is its replacement by shaking in saline. In the latter case the chloride has risen to a higher value than initially. This is not unexpected considering that the chloride content of the saline was higher than that of serum.

The "blob" effect

At Dr Roughton's suggestion a series of three experiments was carried out to test whether shortening of the contact time between corpuscles and phosphate had any effect. The first, represented by curve *A*, Fig. 2, was the control without corpuscles. In the second experiment (curve *B*) 0·5 unwashed pig corpuscles was added to, and mixed with, the phosphate in the boat, and allowed 7 min. for equilibration. The usual small acceleration is seen. The third, for curve *C*, was similar to the second except that, by placing the tip of the pipette under the surface of the solution and running them out slowly, the corpuscles were made to remain in the form of a "blob" (see Fig. 3). The minimum contact was thus made between corpuscles and phosphate, and the effect of this contact was lessened by reducing the equilibration time to the shortest practicable—2½ min. Curve *C* shows that, when the corpuscles did not come into contact with the phosphate until the beginning of the run, a greater acceleration (fourfold at 18°) was obtained in the first 20 sec.

Curve *D* shows for comparison the effect of an enzyme preparation representing a small fraction of the enzyme obtainable from the corpuscles used for each of the other curves. The same results were obtained with sheep corpuscles. This phenomenon will be referred to as the "blob

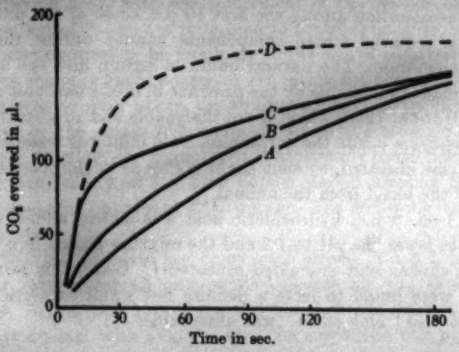

Fig. 2. The "blob effect". CO_2 output. 15°. Quantities as for Fig. 1. *A*, control without corpuscles; *B*, with corpuscles pre-mixed; *C*, with corpuscles unmixed (in "blob" form); *D*, with enzyme solution for comparison.

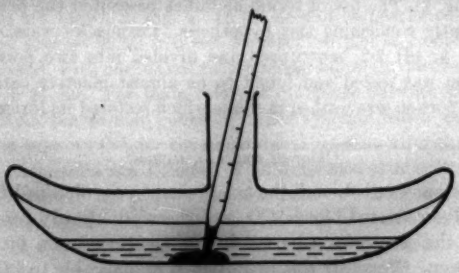

Fig. 3. The "blob" technique.

effect". Reducing the contact time between corpuscles and phosphate to a minimum would lessen the possibility of exchange of Cl' with HPO_4'': hence there could have been no chloride deficiency. Further, as there was no Cl' in the buffer a high concentration gradient of chloride was secured, assuring best conditions for the interchange with bicarbonate. Yet the acceleration obtained (curve *C*) still fell far short of that obtainable with the corresponding amount of isolated enzyme.

Serum as buffer

Other buffers were tried: β-glycerophosphate both at room temperature and at 0·5°, and sodium cacodylate at room temperature. In each case the effect was similar to that obtained with phosphate—a definite but small acceleration during the first 10 p.c. or so of the process only. A buffer is required whose components cannot diffuse through the corpuscle membrane. The natural buffer is serum, and the attempt to use it was made practicable by the presence in it of a carbonic anhydrase inhibitor [Booth, 1938] which is not dialysable and presumably cannot reach the enzyme inside the corpuscles. This inhibitor lessens the effect of any enzyme liberated, by such slight laking as might occur during the drawing of the blood from the animal.

About 1 ml. 8 p.c. hydrochloric acid was slowly added to 100 ml. pig serum to lower the *p*H to 7·2 and the mixture placed in a tall bottle which was shaken and evacuated repeatedly. Using this serum at 18° corpuscles were found to have a definite but small catalytic effect on CO_2 output. No "blob effect" was observable.

Further proof of chloride-phosphate interchange

The chloride-phosphate interchange hypothesis explained the "blob effect" (Fig. 2). The use of serum as buffer presented the possibility of independently confirming this hypothesis. Corpuscles were washed in phosphate at *p*H 7·2, centrifuged and divided into two portions. The one portion was tested and found to be almost inactive catalytically. The other portion was washed in saline which restored its (original small) activity.

Chloride and CO_2 uptake

The corpuscles used in all the experiments were obtained from what corresponds to arterial blood. CO_2 had therefore already been evolved once, and the corpuscles were not in the ideal condition for a second CO_2 evolution. Obviously this limitation is inapplicable to CO_2 uptake. An uptake experiment was carried out at 18° with 4 ml. 0·2*M* phosphate buffer *p*H 7·4 and 0·5 ml. corpuscles. During CO_2 uptake by blood Cl' diffuses *into* the corpuscles. Therefore 0·5 ml. 10 p.c. sodium chloride was added to the buffer solution. This supply of external Cl' together with the low Cl' state of the corpuscles at the beginning of the experiment should provide good conditions for the chloride shift. An initial acceleration was observed, but after 15 sec. the rates with and without corpuscles were identical. Part at least of this acceleration was caused by formation

of carbamino hæmoglobin [Ferguson & Roughton, 1934]—a rapid reaction.

Clearly some other factor than the chloride capacity must also be operative in limiting the catalytic activity of dilute corpuscle suspensions *in vitro*. Having dealt with one component of the system—HCO_3' and its exchange with Cl'—we are prompted to consider another, namely the H^+ which also requires to be available at the site of catalysis. In these experiments the only rapid source of H^+ inside the corpuscles is hæmoglobin, whereas *in vivo* about an equivalent amount is produced by oxygenation of the weak acid hæmoglobin to the stronger acid oxyhæmoglobin. Further, the hæmatocrit is necessarily much below normal. Hence the availability of H^+ may be the second limiting factor. The converse arguments apply to CO_2 uptake. The following experiments clarify this view.

About 300 ml. oxalated pig blood were placed in a 3 l. bottle. The bottle was rotated for 45 min. while CO_2-free air was slowly passed through it. After this procedure the blood would be practically in gaseous equilibrium with CO_2-free air. The corpuscles and plasma were then separated by centrifuging and the corpuscles washed three times in saline. CO_2 uptakes were determined for these corpuscles resuspended in the plasma, in saline and in glucose, and for these solutions alone. The results are shown in Fig. 4. In the case of corpuscles suspended in plasma the process takes 570 sec. to reach 90 p.c. of completion. On the other hand, with corpuscles suspended in saline the process is 90 p.c. complete in 97 sec. The main relevant difference between plasma and saline is that the former contains buffer systems which enable it to continue to take up CO_2 by the uncatalysed reaction after the reactions involving corpuscles are finished. This difference is clearly shown by the uptake curves for plasma and for saline without corpuscles. The HCO_3' formed inside corpuscles suspended in plasma or saline (when CO_2 is taken up) can exchange with Cl' from these solutions. The part played by this exchange is strikingly shown by comparing it with the CO_2 uptake curve for corpuscles suspended in glucose solution in which no such HCO_3'-Cl' exchange is possible. The total CO_2 taken up is much less than when Cl' is available, and those reactions which do occur are 90 p.c. complete in 44 sec. The course of CO_2 uptake by glucose alone is the same as for saline alone. The rate for oxalated plasma alone is unnaturally fast: this is evidently owing to the presence of carbonic anhydrase for the addition of cyanide reduced it. This complication, however, does not affect the main conclusions except to make the

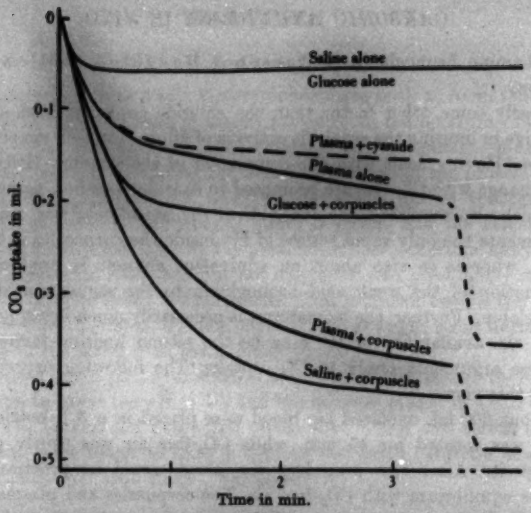

Fig. 4. CO_2 uptake by plasma, by saline and by glucose solution with and without corpuscles. 0°. Gas phase composed of O_2 at 15 cm. Hg and CO_2 at 1·25 cm. Hg. Liquid phase, total volume 4·2 ml., composed of 0·5 ml. corpuscles + 3·7 ml. 1 p.c. saline, 6 p.c. glucose, or plasma; or of 0·2 ml. saline + 4·0 ml. solution.

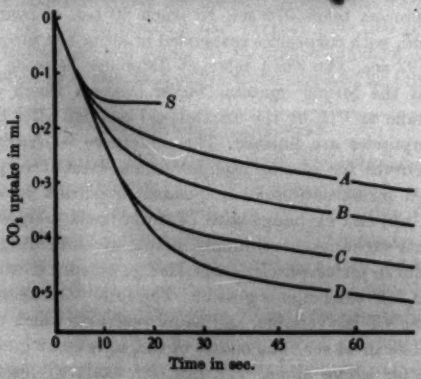

Fig. 5. CO_2 uptake by corpuscles in serum. 18°. 6 p.c. CO_2 added to air at 1 atm. 5 ml. pig serum + x ml. corpuscles + 0·8 − x ml. saline. *A*, control without corpuscles; *B*, with 0·3 ml. corpuscles; *C*, with 0·5 ml. corpuscles; *D*, with 0·8 ml. corpuscles; *S*, 5·8 ml. saline alone.

difference between rates with and without corpuscles appear untruthfully small.

Another CO_2 uptake experiment was done with varying proportions of corpuscles suspended in serum. This experiment is not quantitatively comparable with that in Fig. 4, as the blood was not so carefully freed from CO_2 and the experiment was done at room temperature. The results, shown in Fig. 5, indicate in forcible manner that each corpuscle is only responsible for a given amount of CO_2 uptake. The initial rapid rate, which represents the greatest possible CO_2 uptake velocity observable manometrically, is prolonged in the presence of corpuscles and the several amounts of corpuscles each produce this maximum velocity, but the greater amounts maintain it longer. After this burst of catalytic activity the rates are in all cases the same as that for the run without corpuscles. A capacity effect must be operative. This cannot be connected with HCO_3' or Cl' but is probably the accumulation of H^+ inside the corpuscles which nevertheless would not raise the concentration gradient to a high enough value to effect a rapid migration to the external buffer.

Conclusion

Under suitable conditions red blood corpuscles can take up CO_2 very rapidly. The exact rate cannot be determined manometrically since it at least reaches, and probably exceeds, the maximum rate of the apparatus. Each corpuscle only catalyses the hydration of a limited amount of CO_2, which amount depends upon two factors: (*a*) the removal of HCO_3' by exchange with Cl' from outside, and (*b*) the removal of H^+ by inside buffering. In these experiments the only significant internal buffer is hæmoglobin, but *in vivo* the reduction of oxyhæmoglobin to the weaker acid reduced hæmoglobin results in further removal of H^+. Intact corpuscles cannot catalyse the ordinary process of CO_2 uptake by the buffer in which they are suspended, for the continuance of the rapid (catalysed) reaction is dependent upon the quick removal of H^+ from inside the corpuscles, and this cannot be appreciably affected by the buffer outside. These conclusions hold in converse sense for the evolution of CO_2 from solutions containing HCO_3'. *In vivo* there is ample Cl' for exchange with HCO_3', but as far as buffering is concerned the application to events *in vivo* also holds unless the permeability of the corpuscle membrane is considerably greater to H_2CO_3 or to OH' *in vivo* than *in vitro*.

Summary

1. Intact red blood corpuscles, although rich in carbonic anhydrase, show no activity *in vitro* when tested by the standard manometric method. Two enzyme-substrate accessibility factors are studied.

2. The corpuscle Cl′ available for exchange with HCO_3' during CO_2 output (chloride shift) is exhausted by exchange with HPO_4'' during the equilibration preceding each carbonic anhydrase estimation.

3. On eliminating this factor, by using serum as buffer, corpuscles are active, but each corpuscle only performs a limited amount of catalysis.

4. The second limiting factor is probably buffer capacity of the corpuscles, i.e. *quick* supply and removal of H^+ ions. *In vivo* this is increased by oxygenation and reduction of hæmoglobin coincident with the CO_2 reactions.

I wish to thank Dr F. J. W. Roughton for criticism and suggestions during the progress of this work, and for advice in presenting the results.

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ACTION OF ADRENALINE, ACETYLCHOLINE AND OTHER SUBSTANCES ON NERVE-FREE VESSELS (HUMAN PLACENTA)

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(*Received* 4 *April* 1938)

Recent years have witnessed a rapid progress of knowledge concerning the nature of the chemical transmitters of neuromuscular excitation, and the manner in which the arrival of impulses at the nerve endings causes the release of these substances. Concerning the mode of action of these substances on effector cells, such as muscle cells, on the other hand, knowledge has advanced but little. There is, in the fully developed animal, a striking and specific localization of the action of adrenaline, when artificially applied, to cells which are innervated by adrenergic nerves, and of the action of acetylcholine to cells innervated by cholinergic nerves, though, even in the adult, it is not certain that the action does not extend to some cells which do not receive the corresponding innervation. For example, there are arteries involved in the general vasodilator response to an injection of acetylcholine, for which a corresponding cholinergic innervation has not been demonstrated. On the whole, however, the correspondence is in each case so close as to raise the question whether the response is due to some property or structure conferred on the muscle cell as the result of the particular innervation, or whether it is an inherent property, possessed by the cell before innervation, which the nerve merely uses for the chemical transmission of its effects. Elliott [1905], considering the meaning of the close correspondence between the effects of adrenaline and those of sympathetic nerves, suggested that the union of the sympathetic nerve with the muscle fibre caused the development in the latter, probably in relation to the nerve ending, of a special structure, neither nervous nor contractile, which he referred to as the "myoneural junction", and which he regarded as

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possessing the specific sensitiveness to adrenaline. When this structure had once developed as the result of innervation, degeneration left it intact, and the cell still sensitive to adrenaline. Langley [1905], on the other hand, regarded the persistence of the response to adrenaline after denervation as indicating that the specific sensitiveness was not the result of innervation, and, in further support of that conception, put forward observations on the response to adrenaline of the smooth muscle in the amnion of the developing chick, which has no nerve supply. These observations have been more recently confirmed by Baur [1928], using an improved technique. Baur obtained definite responses of the amniotic muscle fibres, not only to adrenaline, but to a number of other substances, some causing contraction, others inhibition of the spontaneous activity.

In addition to these experiments on the avian amnion, observations have been recorded on the effects of adrenaline and other substances on mammalian fœtal structures, such as the vessels of the umbilical cord and placenta, which apparently lack any nerve supply. Thus Schmitt [1922], having satisfied himself by histological examination that the placenta contains no nerves, made an extensive study of the actions of a number of substances on its perfused blood vessels. He observed constriction in response to histamine, post-pituitary extract (pituglandol) and barium chloride and dilatation with amyl nitrite, but detected no response to adrenaline. With isolated portions of vessels he observed, in a few instances, weak contractions in response to adrenaline in strong concentrations, but drew the conclusion that the absence of any strongly marked sensitiveness of the placental vessels to adrenaline is connected with their lack of innervation—a conclusion in general conformity with Elliott's suggestion.

Several later investigators have described a constrictor effect of adrenaline on the blood vessels of the human placenta and umbilical cord [Kosaka6, 1927; Budelmann, 1929; Baur *et al.* 1929; Küstner & Siedentopf, 1930; Ueda, 1931]. Ueda [1932] could not detect this action of adrenaline on the vessels of placentæ obtained before the sixth month, but observed it in those from stages of pregnancy later than this. This change, however, cannot be attributed to the appearance of nerves, since many attempts to demonstrate these histologically have failed to reveal them even in the full-term placenta [cf. Schmitt, 1929; Baur *et al.* 1929; Guarna, 1934].

There is some conflict of evidence as to whether the action of acetylcholine on the heart can be detected before the vagus fibres have made

connexions with the muscle cells. As early as 1880 Krukenberg had observed that muscarine, so closely resembling acetylcholine in effects of this type, had no action on the heart of the embryo chick, and this finding was later confirmed by Pickering [1893]. Armstrong [1935] states that even comparatively large doses of acetylcholine have no inhibitory action on the embryonic heart of the fish *Fundulus*, before it has been functionally innervated by the vagus. Cullis & Lucas [1936], on the other hand, find that acetylcholine causes a slowing of the rhythm of the aneural heart of the early chick embryo, and that this effect is prevented by atropine. Baur [1928] described actions of acetylcholine on the muscle of the chick amnion, but the responses, like those of the placental vessels which I describe later, were not very pronounced.

Methods

Human placentæ at full term were used throughout[1] and in most cases prepared within ½–1 hr. after the delivery. Cannulæ were inserted into the two umbilical arteries 5–10 cm. from the placenta, and connected to a Dale-Schuster perfusion pump.

As perfusion fluid Locke-Ringer solution of the following composition was used: NaCl 0·9 p.c., KCl 0·042 p.c., $CaCl_2$ 0·024 p.c., $NaHCO_3$ 0·1 p.c., glucose 0·1 p.c. In several experiments the Locke solution was mixed with 5 p.c. hæmoglobin solution, prepared by the method of Amberson & Höber [1932], as modified by Brown & Dale [1936], in the proportion 5–10 parts to 100 parts of Locke. The addition of hæmoglobin solution regularly caused a large increase of the peripheral resistance and a corresponding rise in the perfusion pressure. If the proportion of hæmoglobin solution was raised to 20 : 100 the vaso-constriction was sometimes so strong as to render further perfusion impossible. In some instances the hæmoglobin-Locke solution seemed to improve the reaction of the vessels to the substances added, but in others the reactions and the time of survival (as measured by the reactivity) seemed to be just as favourable with plain Locke solution. Œdema was never observed during the perfusion, whatever fluid was used.

The temperature of the perfused solution, as measured by a thermometer near the cannula tip, was kept at about 37° (36–38°) and the placenta was placed in a thermostat with a temperature of about 35°. Greater variations in temperature were avoided, since it was observed, in confirmation of Schmitt [1922] and others, that the placental vessels

[1] I am much indebted to the Superintendent and the Sisters of the Maternity Ward of the New End Hospital, Hampstead, for supplying the placentæ.

are sensitive to temperature changes, constricting at lower and dilating at higher temperatures.

Probably on account of the low temperature, the vessels of the placenta at the beginning of the perfusion were strongly contracted, and a high pressure was necessary to start the perfusion. When the placenta became warm and the flow through it free, the perfusion rate reached a fairly steady level within about a further 15 min. The rate of perfusion in different preparations has been between 10 and 60 c.c. per min., varying with changes in the arterial pressure produced by adjusting the rate and stroke of the pump, the condition and size of placenta and the composition of the perfusion fluid. Such rates were obtained with arterial pressures of 40–100 mm. Hg. According to Haselhorst [1929] the physiological pressure in the umbilical arteries varies between 46 and 110 mm. Hg with an average of 70–75 mm. The preparations have been supplied with fresh solution during the whole experiment. The plain Locke solution was not oxygenated, but in one case in which hæmoglobin-Locke solution was used, this was oxygenated in advance by bubbling oxygen through it. Differences in reaction referable to the state of oxygenation of the solution have not been observed. It is to be remembered that the fluid passing through the umbilical arteries is normally venous; oxygen has been found [Schmitt, 1922] to cause the arteries to contract strongly.

The time elapsing between delivery and the start of the perfusion does not, within limits, seem to exert a noticeable influence on the reactions to adrenaline and other substances. Thus, some quite fresh and still warm preparations, rapidly brought to a constant venous outflow, have reacted feebly to most of the substances added, whereas other preparations, kept for several hours at room temperature or in the ice chest, have reacted well. These wide variations in sensitivity might, on the other hand, provide an explanation for the varying results reported by different investigators. Again, a high sensitivity of the placental vessels to one vaso-motor agent did not necessarily imply an equal sensitivity to others.

The substances which have been tested with regard to their action on the placental vessels have either been injected in volumes of 0·05–1 c.c. into the rubber tubing near the arterial cannulæ, or, in some instances, have been added, by infusion from a Mariotte burette, to the stream of fluid on the intake side of the pump. Information has thus been obtained as to the actual concentrations of the active substances reaching the placenta, by determining the rate of such addition and the perfusion

rate concurrently. The substances have been dissolved in Locke solution or, in some cases, in plain physiological saline, acidified with HCl to *p*H about 4. Control injections have been carried out as a routine and have shown, in the majority of experiments, that volumes of up to 1 c.c. of either of these solutions, injected at room temperature, have not caused any significant vascular reaction, as judged by the perfusion pressure and perfusion volume.

The rate of perfusion has been recorded either by direct determination of the amount of fluid leaving the umbilical vein during a measured time, or by means of the Gaddum outflow recorder [1929]. The perfusion rate and pressure have generally been constant or shown only smooth changes, but in some instances the pressure curve has been irregular, indicating spontaneous variations in the muscular tone.

Results

The following substances have been examined for vascular effects on the placenta: adrenaline, acetylcholine, vasopressin, histamine, adenosine and prostaglandin; in addition, some observations have been made on the actions of ergotoxine, cocaine, atropine, eserine and barium chloride.

(*a*) *Adrenaline* (*ergotoxine, cocaine*). As already mentioned, Schmitt [1922] was unable to observe any action of adrenaline even in strong concentrations, on the human placenta perfused under a constant, non-pulsatile pressure, whereas other investigators have observed an effect.

In most of my experiments adrenaline produced a definite vaso-constrictor effect (see Table I), appearing as a rise in the perfusion pressure and diminution of the rate of the venous outflow. Reactions have been observed on direct injection of amounts as small as 2 μg., and in some instances 5 μg. have produced a considerable effect (Fig. 1 A). The most common type of response is illustrated in Fig. 2. Occasionally, however, only very small reactions or none at all were obtained with doses up to 0·5 mg. This failure to react has been observed even in quite fresh preparations, which showed good vaso-constrictor reactions to other substances. The lowest effective concentrations of adrenaline, given by infusion, have been of the order of 1 : 2·5 millions, though in several experiments the actual concentration necessary to produce a reaction has been considerably higher. In no case has vaso-dilatation been observed.

In those experiments where a definite reaction to adrenaline has been obtained it has always been possible to abolish it with ergotoxine (Fig. 1 D),

which itself caused a long lasting vaso-constriction when added in sufficient concentrations. Abolition of the adrenaline effect has been observed with a dose of 0·05 mg. ergotoxine, which did not cause a noticeable effect by itself, whereas 0·25 mg. or more caused vaso-constriction.

Fig. 1. All figures are from the pump-perfusion of the human placenta. Where not otherwise stated the record is of the perfusion pressure. A, 5 μg. adrenaline; 10 min. before B 1 mg. cocaine hydrochloride. B, 5 μg. adrenaline; 3 min. before C 1 mg. cocaine hydrochloride. C, 5 μg. adrenaline; between C and D 0·25 mg. ergotoxine ethanesulphonate. D, 5 μg. adrenaline.

Since cocaine is known to enhance the actions of adrenaline, the effect of this substance on the response to adrenaline was tested in some experiments. According to Burn & Tainter [1931] cocaine exerts its sensitizing effect on the adrenaline actions by some process occurring peripherally of the nerve endings. It might be expected, therefore, that cocaine would produce its characteristic action even on nerve-free muscle, and in two out of eight experiments such an action has been demonstrable (Fig. 1 C), and a doubtful effect in two others. In my experiments the best effect was obtained with a dose of about 2 mg. of cocaine hydrochloride, injected 2–5 min. before the adrenaline. In doses higher than 5 mg. cocaine regularly caused vaso-dilatation, and after doses of 20 mg. and more complete insensitivity to all substances tested has been observed, apparently due to paralysis of the smooth muscle. With the small doses required for sensitization, no definite direct effect of cocaine has been observed.

Fig. 2. Upper curve: venous outflow. Lower curve: perfusion pressure. At arrow 10 μg. adrenaline.

These experiments have thus shown that the action of adrenaline on a nerve-free preparation is abolished by ergotoxine and may be enhanced by cocaine, as on ordinary, sympathetically innervated smooth muscle. These substances must, therefore, like adrenaline, have their point of attack in the muscle cell itself.

(*b*) *Acetylcholine* (*atropine, eserine*). Whereas the action of adrenaline could be demonstrated with a fair degree of regularity, it has been

Fig. 3. *a*, 0·2 mg. acetylcholine; *b*, 25 μg. adrenaline.

Fig. 4. A: *a*, 0·2 c.c. acid saline; *b*, 10 μg. acetylcholine. B, 0·2 mg. eserine. C, 10 μg. acetylcholine + 0·2 mg. eserine.

possible to obtain definite and regular effects of acetylcholine only on an occasional preparation. In some cases a small decrease in pressure, corresponding to a vaso-dilatation has been observed (Fig. 3 *a*). This effect has always been very weak, however, even if the existing vascular tone has been good. During the period of increased pressure due to an injection of adrenaline, the effect of acetylcholine was sometimes seen as a depression in the curve. In certain preparations, however, a rise in

pressure, due to vaso-constriction, has been produced by acetylcholine. This effect has varied within wide limits, from a just perceptible retardation to an almost complete arrest of the perfusion. Strong reactions of this kind have been obtained even with comparatively small doses of acetylcholine, of the order of 5–25 μg. (Fig. 4). The pressor response was occasionally enhanced in strength and duration by a previous or a simultaneous dose of eserine (0·2 mg.). It is noteworthy that this pressor effect of acetylcholine has not been obtainable repeatedly on the same preparation, unless sufficient time was allowed to elapse between successive doses. When, for instance, a second, identical dose was injected, immediately after the definite effect of a first dose had passed off, it produced only a very weak response, or none at all; only some 15–20 min. later would a second dose produce a similar effect to the first. Eserine by itself, in the amounts used (0·1–0·2 mg.), caused a moderate vaso-constriction.

These effects of acetylcholine were readily abolished by atropine, which thus produces its typical effect independently of the presence of nerves. In Fig. 5 the prevention by atropine of the pressor response to acetylcholine is illustrated. Atropine itself caused no effect in amounts of 0·1–1 mg.

Fig. 5. A, 25 μg. acetylcholine. B: a, 1 mg. atropine; b, 25 μg. acetylcholine.

In view of these varying effects of acetylcholine on placental vessels, it is of interest to note that Baur *et al.* found variations in the response of human umbilical vessels to pilocarpine, which in some cases caused vaso-dilatation and in others vaso-constriction. Ueda [1931] reports that lower concentrations of acetylcholine produce vaso-dilatation in placenta, and higher concentrations vaso-constriction. These findings are not supported by my results, in which no relationship between dose and action can be observed. Moreover, the response to acetylcholine has been absent, or insignificantly weak, in the majority of experiments.

Incidentally, it was observed that a previous dose of quinine (0·2–1 mg.) caused an increase of the pressor response to acetylcholine (25 μg.). A sensitizing effect of quinine, on the response of various test objects to acetylcholine, has recently been described by Kahlson & Uvnäs [1938].

(c) *Histamine.* Schmitt [1922] found histamine very active as a constrictor of the human placental vessels, and the same observation

was made for the umbilical vessels by Baur *et al.* [1929]. The last-mentioned authors found, however, considerable variations in the response to histamine, without any apparent reason. My own experience confirms these observations, though in most of the experiments the action has been weak, even with doses of 10–20 μg. The only effect observed has been vaso-constriction. It was also repeatedly noted that a previous dose of histamine diminished the response to a subsequent dose, if the interval between the injections was short (Fig. 6). This effect may possibly

Fig. 6. *a*, 20 μg. histamine. *b*, same dose 6 min. after the previous one.

be of a similar nature to the desensitizing effect of a persistent concentration of histamine, as found by Barsoum & Gaddum [1935] on the guinea-pig's intestine.

(*d*) *Vasopressin.* Previous investigators have observed either vaso-constriction or no action with pituitary preparations, except Kosaka é [1927], who regularly obtained vaso-dilatation after a short vaso-constriction. In the experiments recorded here pitressin (Parke, Davis and Co.) has been used, and in most cases produced vaso-constriction, though in a few instances no effect was obtained even with doses as high as 10 I.U. The vaso-constrictor effect has been small even with doses of 2–5 I.U., and the response has consisted of a slow rise followed by a slow fall in pressure (Fig. 7), different in type from that obtained with adrenaline.

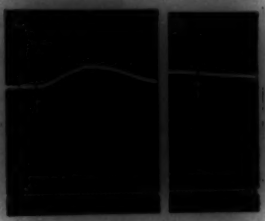

Fig. 7. A, 2 I.U. pitressin. B, 4 I.U. 8 min. later.

The characteristic diminution of the response to a second and following doses, administered within a short time after the first dose, well known from the action of posterior pituitary lobe extract on the general blood pressure, could be demonstrated also on the placental vessels.

It is of interest that in one instance, where 2 I.U. of pitressin caused a definite action, the preparation was insensitive to adrenaline even in a dose of 0·5 mg., whereas in another case pitressin was without action in 10 I.U. and adrenaline well active. In two other experiments both substances produced a reaction, and it could be shown that adrenaline was active even when the vessels were "refractory" to pitressin, after the first dose.

(*e*) *Adenosine.* Adenosine does not seem to have been tested previously on normally nerve-free preparations. On innervated vessels the responses are mostly vaso-dilatation [Bennet & Drury, 1931], but vaso-constriction has been observed on the lung vessels of the rabbit and other animals.

The effect on the placental vessels was not constant in all preparations. In two experiments doses of 0·1–0·3 mg. produced no perceptible reaction, whereas 0·4–0·6 mg. caused a rise in perfusion pressure (Fig. 8 B).

Fig. 8. A, 10 mg. sodium nitrite. B, 0·5 mg. adenosine.

This effect could be readily repeated in the same preparation. In view of the similarity found by Drury between the action of adenosine and nitrites, it was of interest to find that, in preparations in which adenosine caused vaso-constriction, the injection of sodium nitrite in an amount of 5–10 mg. caused a pronounced vaso-dilatation. In one experiment, however, the response to 0·1–0·3 mg. adenosine was a definite decrease in pressure, indicating vaso-dilatation, and no certain effect was obtained with 0·4–0·6 mg.

Comparing the reactions to acetylcholine and adenosine of the lung vessels of the rabbit and of the vessels of the human placenta, it seems noteworthy that both organs respond to these substances with vaso-constriction, which is an exceptional reaction.

(*f*) *Prostaglandin.* This substance has recently been described by the present writer [Euler, 1936], who found it in prostatic secretion and extracts of the prostate of man and certain animals. Normally it produces

a long persistent lowering of the arterial pressure when injected intravenously into rabbits, cats or dogs, also after atropine. It produces an increased activity of the intestine and uterus of a number of animals. The injection into the perfusion fluid of 1–2 units (1 "unit" causing a fall of 30 p.c. in the blood pressure of a rabbit of about 2·5 kg.) regularly produced a vaso-constriction of long duration in the placenta (Fig. 9), a type of effect which has not been previously observed. Since the active substance has not been chemically isolated, the activity of the extract was tested after inactivation of the prostaglandin by treatment with *N* HCl for 10 min. at 100°, in order to exclude the action of a possible contamination with histamine. After neutralization, however, a corresponding dose now produced no significant action, so that the action before hydrolysis was not due to histamine.

Fig. 9. Upper curve perfusion rate, lower curve perfusion pressure. At arrow 1 unit prostaglandin.

(*g*) *Barium chloride.* In the cases where barium chloride was tested it produced a vaso-constriction, even when the vessels did not respond to adrenaline, as observed already by Schmitt [1922].

The main results have been collected in Table I, which shows the effects of the different substances alone and in relation to others.

TABLE I. Effects

Substance	Definite	Weak or absent	Constriction	Dilatation	Dose
Adrenaline	11	5	13	—	2–500 μg.
Acetylcholine	2 (constr.)	10	5	3	5–500 μg.
Histamine	2	3	3	—	5–20 μg.
Vasopressin	1	4	3	—	2–10 I.U.
Adenosine	3	—	2	1	0·1–0·6 mg.
Prostaglandin	3	—	3	—	0·5–2 units
Ergotoxine	—	—	3	—	0·02–1 mg.
Cocaine	—	—	—	6	1–20 mg.
Atropine	—	2	—	—	0·1–1 mg.
Eserine	—	—	4	—	0·1–0·2 mg.
Quinine	—	1	—	—	0·1–1 mg.
$NaNO_2$	1	—	—	1	5–20 mg.
$BaCl_2$	2	—	2	—	5–50 mg.

Modifying effects of other substances

	Increased	Abolished	Unchanged
Adrenaline by cocaine	2 (+2)	—	4
Adrenaline by ergotoxine	—	4	—
Acetylcholine by eserine	1	—	3
Acetylcholine by atropine	—	1	—
Acetylcholine by quinine	1	—	—

Discussion

My observations are fully in accord with those of earlier observers in showing that the smooth muscle of the placental vessels possesses a natural tone, sensitive to oxygen tension, as earlier shown by Schmitt [1922] and by Baur *et al.* [1929], and especially to temperature. There are no nerves to affect this tone, and there is no ground for assuming any hormonal control of it. My experiments with substances which might function as hormones, such as adrenaline and vasopressin, show that the reactions of the placental vessels to these, as to other substances affecting their tone, though definite, are weak and variable in comparison with those of other blood vessels. It seems probable that, while the placenta is still functional and protected from temperature changes, the only condition likely significantly to affect the tone of its vessels is the oxygen supply.

It will have been seen that both the known transmitters of nervous excitation, adrenaline and acetylcholine, have effects on the tone of the placental vessels, those of adrenaline being the more regular. These effects, like those earlier observed on the amniotic membrane, show, indeed, that these transmitters are not without action on plain muscle cells which have never been innervated. They do not, on the other hand, exclude the possibility that connexion with nerve endings may confer, on muscle cells which acquire it, a sensitiveness of higher degree and greater specificity for one or the other of these substances. Indeed, the relatively weak and somewhat variable reaction exhibited by the placental vessels might well be regarded as representing a primitive power of response to these transmitters. In somatic arteries which acquire a nerve supply, this power of response to adrenaline would accordingly not be evoked as an entirely new property by union with nerve fibres of the sympathetic system, but would, nevertheless, be greatly enhanced thereby and given a definite direction. The rival conceptions of earlier investigators might, on these lines, be harmonized.

In some of the experiments acetylcholine showed a definite constrictor action on the placental vessels—an action which, like its other peripheral effects, is readily abolished by atropine. It is worthy of note that such a vaso-constrictor action of acetylcholine in small doses has elsewhere been regularly observed on the pulmonary vessels of the rabbit [Euler, 1932]; and it is tempting to correlate this similarity of response with the fact that the arteries of both the placenta and the lungs are conveying venous blood, and are structurally adapted to withstand only moderate

pressures. There is a similar resemblance between the response of the placental and the pulmonary vessels to adenosine, which causes constriction of both, in contrast to its general vaso-dilator action. These vaso-constrictor effects of acetylcholine and adenosine on the placental vessels are not seen in all experiments. Both, on occasion, have caused vaso-dilatation, and the question arises whether their actions, in causing an increase of the resistance to perfusion, are necessarily, or entirely, upon the blood vessels. If the placenta contained extravascular contractile tissue, responding to these substances by contraction, its reaction might cause an obstruction to the flow stimulating a vaso-constriction, and even, perhaps, masking a concomitant relaxation of the vascular walls themselves. In a tissue like that of the placenta it is practically impossible to determine, by histological examination, whether a spindle-shaped cell is contractile or not. There is, accordingly, no evidence that such extravascular contractile tissue is present, or any ground for assuming, if it were, that its reactions to these drugs would be in a different sense from that of the arteries; and, if its presence were invoked to explain an increase of resistance to perfusion sometimes caused by the generally vaso-dilator acetylcholine, it would leave unexplained the fact that another general vaso-dilator substance, adenosine, may produce a similar obstruction of flow through placentæ on which acetylcholine has no effect.

Though the effects on the placental vessels of the various substances tested must, in the absence of nerves, or any structures associated with innervation, be attributed to direct actions on the plain muscle cells, it is unlikely that they act directly on the contractile elements of these. If two substances both caused vaso-constrictor action by directly stimulating the contractile elements, we should expect to find that their actions would appear with approximately constant relative intensities in different experiments, and that failure to react to either would mean failure with both. This is by no means the case. It would appear, then, that even these primitive cells, with no nerve supply, possess some structure responsible for fixing the chemical stimulants or procuring their access to the contractile elements, and probably for determining the direction in which the contractile function will be affected by them; and that this structure may show independent variations in its fixative power or its permeability for different active substances. In other words, they must possess something corresponding to what Langley termed "receptive substances". It is upon the fixing power or permeability of such that atropine must be supposed to act, when it renders the placental

vessels insensitive to acetylcholine or pilocarpine [Ueda, 1931], and leaves their reaction to other substances unaffected.

Summary

1. The effects of a series of substances on the blood vessels of the perfused human placenta have been observed, with the following results:

Adrenaline. Constriction seen in most experiments with doses of 2 μg. or more, and reproduced with repeated application. Effect sometimes enhanced by cocaine and always abolished by ergotoxine.

Acetylcholine. Usually no effect, but occasionally weak dilatation, or a pronounced constriction enhanced by eserine and abolished by atropine.

Histamine, vasopressin. Constriction, usually weak, a second dose producing a diminished effect or none.

Adenosine. Dilatation or constriction, the latter in cases showing dilatation with nitrites.

Prostaglandin. Pronounced constriction.

2. It is concluded that all these substances, including the chemical transmitters of nervous effects, have direct actions on the muscle cells, but, from the variations in their relative potencies, that these actions are not on the contractile substance directly.

3. The similarity between the responses of placental and pulmonary vessels to certain substances is noted.

It is a great pleasure for me to express my grateful acknowledgement to Sir Henry Dale for the facilities placed at my disposal during this work. I also wish to thank Dr G. L. Brown for much valuable advice.

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J. Physiol. (1938) 93, 144–158 612.015.3:612.766.1

THE EFFECTS OF PROLONGED MUSCULAR EXERCISE ON THE METABOLISM

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(*Received* 2 *May* 1938)

Courtice & Douglas [1936], in experiments on the effect of prolonged muscular work of moderate severity, have shown that though the respiratory quotient may be somewhat raised throughout the period of work, it falls to a low level during subsequent rest, and that this low level may be maintained for many hours and is accompanied by ketonuria. In discussing the significance of these changes of R.Q. in relation to carbohydrate and fat metabolism, they have raised the question whether they are to any degree determined by variations in the activity of the endocrine glands associated with carbohydrate metabolism. It was felt, therefore, that the conditions obtaining during and after muscular work of this type were worth further study from this point of view. I wish to thank Dr C. G. Douglas for the help he has given me, and particularly for acting so frequently as subject.

In the experiments to be described the same exercise was taken as in Courtice & Douglas's experiments, viz. a 10 mile walk on almost level ground at the speed of 4·5 miles per hr., a rate of muscular work which they found, in agreement with the earlier observations by Owles [1930], to be unaccompanied by the accumulation of any material excess of lactic acid in the blood. Mills and Douglas served as subjects, neither being in muscular training, though Mills was more accustomed to long walks. The experiments were always made with the subject in the post-absorptive state. The respiratory exchange was determined by the bag method, the initial values being obtained in duplicate by collecting two successive samples of expired air, each over a period of about 10 min. Blood sugar was estimated in samples of blood obtained by pricking the finger, after the hand had been immersed in warm water for some

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minutes previously to encourage the circulation, by the method of Hagedorn & Jensen [1923] with later modifications as described by Peters & van Slyke [1932], except that for filtering Whatman no. 44 filter paper was used. Urine was tested qualitatively for sugar by Benedict's test, and for ketone bodies by Rothera's test.

Mills differed from Courtice and Douglas in showing a higher initial R.Q. during rest before the exercise, and though the exercise sometimes caused it to fall, it was still much higher than that shown by these two subjects when it was determined after an interval of rest of at least 1 hr. had elapsed since the exercise. These differences are shown in Table I.

TABLE I. Average values for resting subject before and after a 10 mile walk at 4·5 miles per hr.

		Respiratory exchange c.c. per min.		
		CO_2	O_2	R.Q.
Mills:	Before	186	223	0·84
	After	186	230	0·82
Courtice:	Before	181	223	0·81
	After	181	237	0·76
Douglas:	Before	182	229	0·79
	After	176	240	0·73

In correspondence with the higher R.Q. shown by Mills during rest after exercise he never showed ketonuria, though Douglas invariably did so on his normal diet. The difference is probably to be explained by the fact that Mills takes a far larger proportion of carbohydrate in his natural diet than do either of the others.

Glucose tolerance curves

The original experiments on Courtice showed some depression of the tolerance to 50 g. sucrose after exercise. A series of such tests has now been made upon Douglas and Mills after a dose of 50 g. glucose, which is the standard used in most recent work, both clinical and experimental.

The routine of our experiments was as follows:

The subject on arrival in the laboratory, or after a walk of 10 miles and a preliminary rest of at least 1 hr., reclined in a deck chair while two bags of expired air were collected, in order to determine the initial basal R.Q. During the determination of the sugar tolerance the subject sat on a stool, and as he had to help in procuring blood samples he was not at complete rest; and he had also to rise at intervals to produce samples of urine. Two samples of blood were taken to determine the resting blood-sugar level. The subject then took 50 g. glucose, dissolved in

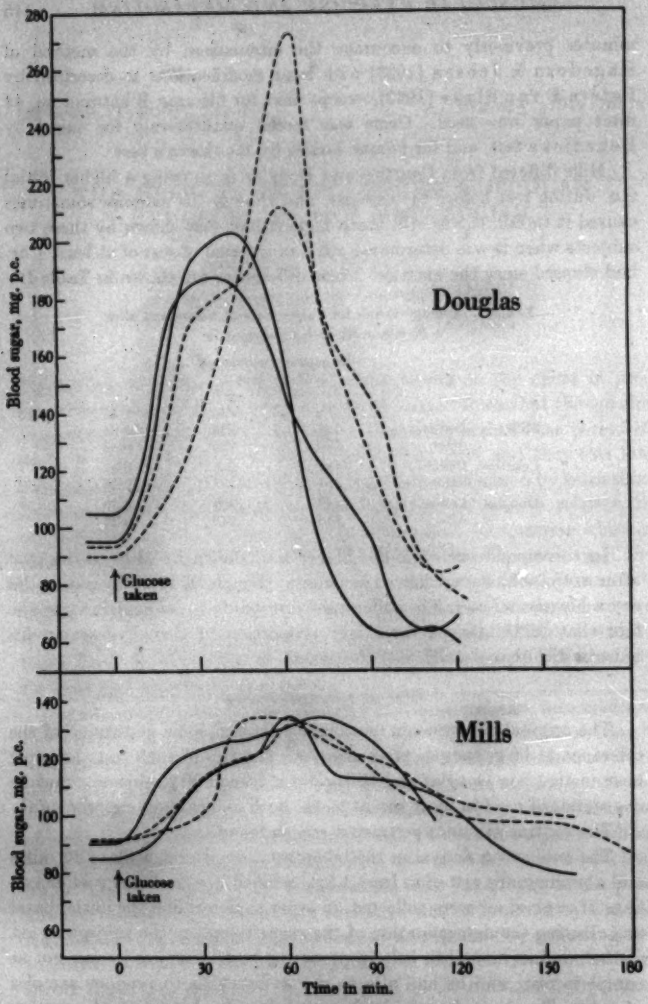

Fig. 1. Effect of ingesting 50 g. glucose upon the blood sugar, before and after walking 10 miles at 4-5 miles per hr. Continuous lines, before walk: broken lines, after walk.

300 c.c. water; and samples of blood were taken at intervals of 15 min. for 2 hr. (In some experiments the interval was lengthened to 20 min. for the second hour and the experiment prolonged for a third hour.)

The results are shown in Fig. 1, continuous lines being curves obtained before a walk and dotted lines after a walk.

In Douglas's case the curves after exercise show all the criteria of reduced tolerance, as did the sucrose tolerance curve of Courtice: the peak is raised and delayed, the return to normal is delayed, and the area, as computed by means of Simpson's rule, is increased by about 60 p.c. The very high peak of one curve is based on a single analysis; as, however, there was some difficulty and delay in obtaining the blood sample, too much stress should not be laid on this value.

The curves obtained upon Mills are of an entirely different type, the blood sugar rising gradually to about 140 mg. p.c. and falling still more gradually towards the resting level; this is the type of curve commonly associated with a high carbohydrate diet. The curves after exercise do not differ noticeably from the resting ones, except that there is a tendency for the initial rise to be more gradual. This same tendency was also shown rather more strikingly in the curves upon Douglas.

This apparent delay in intestinal absorption might perhaps be a result of dehydration, as the glucose solution taken was hypertonic. To test this possibility, three pairs of experiments were performed upon Mills in which the glucose tolerance was determined for one hour only and the results are shown averaged in Fig. 2: *a* is a pre-exercise curve, *b* a post-exercise curve when fluid intake was severely restricted, and *c* a post-exercise curve when abundant fluid was taken. The steeper rise of *c*, as compared with *b*, suggests the possibility that dehydration was partly responsible for the slow rise of the post-exercise tolerance curves in Fig. 1. It should be noted that, in these experiments, Mills showed distinct evidence of lowered tolerance after exercise, which he never did in the earlier experiments.

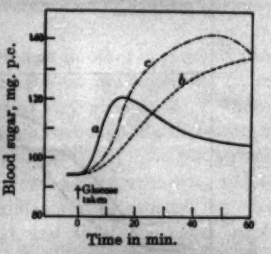

Fig. 2. Mills, post-absorptive. Effect of ingesting 50 g. glucose upon the blood sugar. Each curve is the average of two experiments. *a*, before walking; *b*, after a walk, with fluid intake severely restricted; *c*, after an identical walk, with abundant fluid intake.

Himsworth [1934] has shown that the tolerance is improved by a diet rich in carbohydrate; we therefore tried the effect upon Douglas of

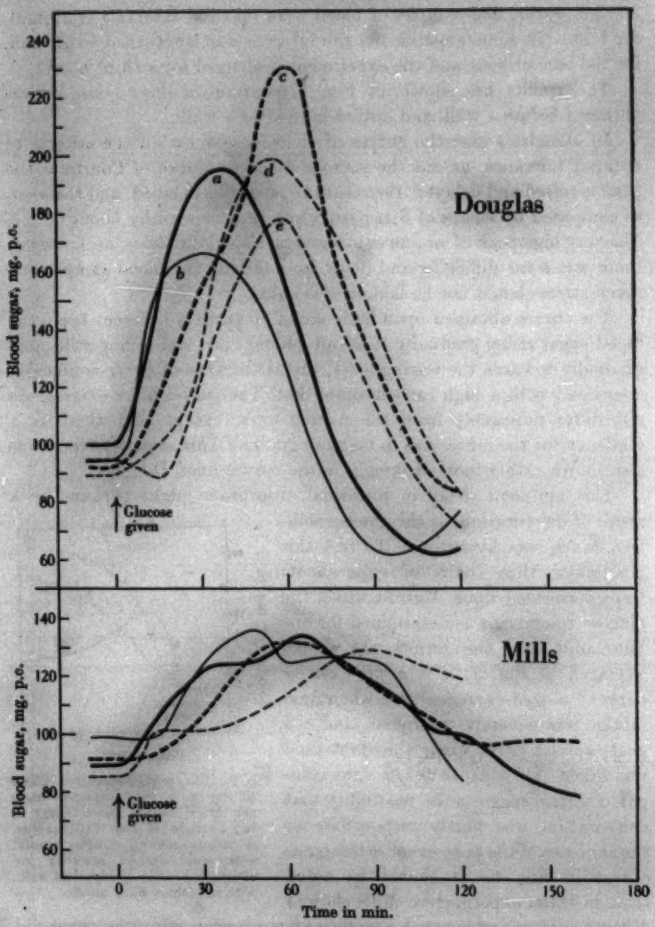

Fig. 3. Effect of ingesting 50 g. glucose upon the blood sugar, before and after walking 10 miles at 4·5 miles per hr., upon normal and abnormal diet. Continuous lines, before walk: broken lines, after walk. Thick lines, normal diet: thin lines, abnormal diet, with the carbohydrate of Douglas increased, and of Mills restricted.

a high carbohydrate diet, and upon Mills of a diet in which the carbohydrate was greatly reduced. In Fig. 3 these curves are shown in thin lines, with the curves obtained upon a normal diet in thick lines for comparison. The curves for Douglas, shown above, each represent the average of two experiments, except that the two curves obtained after exercise upon a high carbohydrate diet, *d* and *e*, are shown separately, as they differ markedly from one another. It is probable that one of these, *d*, should be disregarded, since the subject showed slight ketonuria after exercise, which he did not normally do on the day after a high carbohydrate diet.

The effect of the diet upon Douglas consists in a lowering of the curve without alteration in its time relations, that is, in the well-known improvement of glucose tolerance produced by an increase of dietary carbohydrate. So great is this improvement in curves obtained after exercise that exercise no longer depresses the tolerance; the tolerance in curve *e* is hardly lower than in curve *b*, whether judged by height of the peak or area enclosed beneath the curve; the peak in *e* is certainly later, but this may be due to delay in absorption, and cannot be taken as evidence of impaired tolerance.

Restriction of the carbohydrate in the diet of Mills had no definite effect, either in decreasing the glucose tolerance or in causing exercise to decrease the tolerance.

In some of the later experiments, as well as in other experiments in which we determined the R.Q. after taking glucose, we tested the urine for sugar at intervals. Mills never showed any glycosuria. Douglas showed no glycosuria when living on a high carbohydrate diet except upon the day of curve *d*, when there was a slight trace; upon his normal diet he usually showed glycosuria in pre-exercise experiments, which disappeared after an hour, and in experiments after exercise he constantly showed glycosuria in the second and third half-hours after taking glucose.

We have also performed a few experiments upon the respiratory exchange after taking glucose, before and after a walk; the routine was the same as for the glucose tolerance curves, except that the subject was at complete rest.

The results are shown in Fig. 4. It will be noted that neither subject showed the great rise of R.Q., to nearly unity, which Courtice showed before exercise. It can be calculated, using the Zuntz-Loewy [1920] tables, that after ingesting 50 g. glucose Douglas shows an excess metabolism of only about 5 g. carbohydrate.

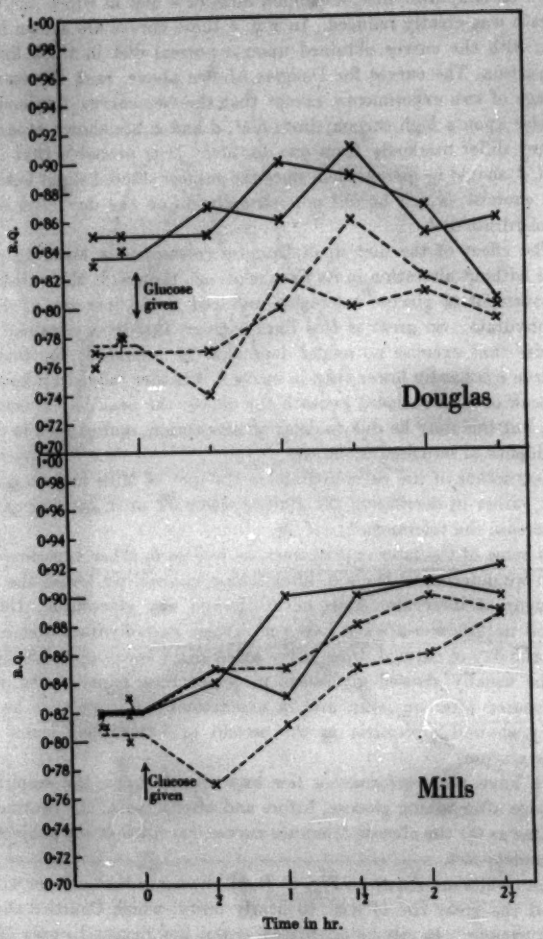

Fig. 4. Effect upon R.Q. of ingesting 50 g. glucose, before and after walking 10 miles at 4·5 miles per hr. Continuous lines, before walk: broken lines, after walk.

Insulin sensitivity

If glucose tolerance is due to the action of insulin, then a reduced tolerance may be due to diminution in the rate of insulin secretion, or, as Himsworth [1935] has suggested, to diminished sensitivity to insulin. In order to test whether the lowering of glucose tolerance resulting from prolonged exercise is associated with diminished sensitivity to insulin, the blood sugar has been estimated after the intravenous injection of insulin. Two samples of blood were first taken to determine the resting level; insulin (Burroughs, Wellcome & Co., made with crystalline insulin) was then injected into a vein in the forearm; and blood samples were taken at intervals of 2–3 min. for about 20 min. to determine the rate of fall of blood sugar, and thereafter at longer intervals, continuing for an hour in the later experiments, in order to investigate the recovery phase.

Fig. 5 shows on the left curves obtained upon Douglas (*D*) and upon Mills (*M*) after 5 units of insulin; there is surprisingly little difference between them, in spite of the very different form of glucose tolerance curve of the two subjects. The difference between their tolerance curves can, therefore, hardly be due to a different sensitivity to insulin produced by their different diets.

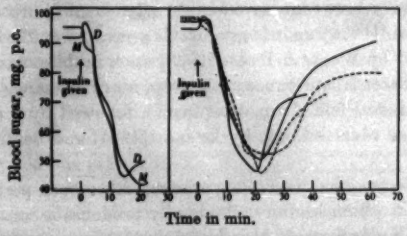

Fig. 5. Effect upon blood sugar of intravenous injection of insulin. On left, 5 units on the subject before exercise: *D*, Douglas; *M*, Mills. On right, 3 units on Douglas. Continuous lines, before a walk. Broken lines, after walking 10 miles at 4·5 miles per hr.

This dose of insulin produced disagreeable subjective symptoms of hypoglycæmia, with tremor, disturbance of vision, and sweating; in later experiments, made on Douglas, the dose of insulin was therefore reduced to 3 units.

Fig. 5 shows on the right four curves, two before a walk in continuous lines, and two after a walk in broken lines. All four agree fairly closely during the steep period of fall of blood sugar, but the blood sugar

appears to return more rapidly towards normal in the pre-exercise curves. Since the sensitivity to insulin is represented by the phase of falling blood sugar, the lowered glucose tolerance after exercise does not seem to be due to lowered sensitivity to insulin.

As has been said, Douglas always developed ketonuria after exercise, though Mills never did. The intravenous injection of 3 units of insulin considerably reduced the Rothera reaction in the urine in Douglas's case in 20 min., and abolished it in about 40 min. Urine samples have twice been collected for 2 hr. after the injection of insulin, and in neither case had ketonuria reappeared, although the R.Q. 1 hr. after exercise was 0·75 and 0·76.

Respiratory exchange after injection of insulin

Observations have also been made upon the respiratory exchange after the intravenous injection of 3 units of insulin. There is a considerable literature upon this subject, which has been summarized by Stammers [1926] and by Richardson [1929], and the general impression gained is that when the initial quotient is low, near 0·71, it is raised by insulin, but that when it is high the effect of insulin is very variable and almost always much smaller. Experiments in which the quotient, although initially not very low, rose considerably after insulin, are described by Dickson *et al.* [1924] upon dogs, by Cammidge & Howard [1924] upon rats, and by Weiss & Reiss [1923] upon rabbits, narcotized with urethane to prevent disturbance of the respiratory exchange by muscular activity. An initial fall of R.Q. after insulin followed by a slow rise has sometimes been observed, e.g. by Lesser [1924] and by Lyman *et al.* [1923].

Most of the recorded human experiments have been performed upon the diabetic, in whom insulin undoubtedly produces a considerable rise of the R.Q. Kellaway & Hughes [1923] have given insulin subcutaneously to a single normal human subject and produced a gradual rise of quotient from 0·73 to 0·92: and Lyman *et al.* [1923], upon giving intravenous doses of 2½–5 units to normal human subjects, have observed a rise of quotient of very varying magnitude, the greatest being from 0·794 to 0·943 and the smallest from 0·847 to 0·877: the maximum quotient was reached after about 30 min., except in one subject in whom after insulin the quotient first fell for 10 or 20 min. In more recent years no human experiments seem to have been recorded, and no conspicuous addition to our knowledge has been made in animal experiments. We had, therefore, very little idea what result to expect from our experiments.

Experimental. After a duplicate determination of the resting R.Q., insulin was injected into a vein in the forearm, while the subject still breathed through valves, and in the earlier experiments continuous collection of expired air was commenced immediately. It was found, however, that the metabolic rate was always raised in the first sample, through the disturbance of the subject during the insulin injection, so 2, and later 5 min. were allowed to elapse after the injection before commencing the respiratory exchange determinations. Since in the earlier resting experiments insulin produced no large change in the R.Q., blood-sugar analyses were performed in the later experiments to make certain that the insulin had entered the blood stream; one blood sample was taken immediately before injecting insulin, and another about 20 min. later, during an intermission of about 10 min. in the respiratory exchange determinations. The blood sugar was never quite as low as one would expect from the curves of blood sugar after insulin; the probable reason is that in these experiments the subject was at almost complete rest, whereas in the experiments upon blood sugar after insulin he frequently changed his position, and therefore presumably consumed more glucose. It is also noteworthy that no subjective symptoms were ever observed after insulin in the experiments upon respiratory exchange, whereas they were constantly observed in the blood-sugar experiments. In one experiment the blood-sugar analysis showed that insulin had not entered the vein, and this experiment was therefore disregarded. Of the experiments in which blood-sugar analyses were not performed, only those have been recorded in which the respiratory exchange behaved as it did in the experiments with blood sugar controlled, that is, one of the curves before and one after a walk.

Fig. 6 shows, above, the quotients obtained in experiments in which insulin was injected 80–95 min. after the end of a walk. It is seen that after insulin after a walk the quotient rises gradually from its low level to a peak, maintained from about the 30th to the 40th min., and that it then falls rather rapidly to the initial level again. The early stages of the rise might be due to the respiratory exchange of combustion of ketone bodies, but since ketonuria has disappeared in 30–40 min., the determination at the peak level cannot be vitiated through this cause. The presumptive high level of carbohydrate metabolism between the 30th and 40th min. may account for the persistence of a low blood sugar during this period.

In the experiments before exercise, shown in Fig. 6, the behaviour of the quotient is less constant in separate experiments, and in one curve

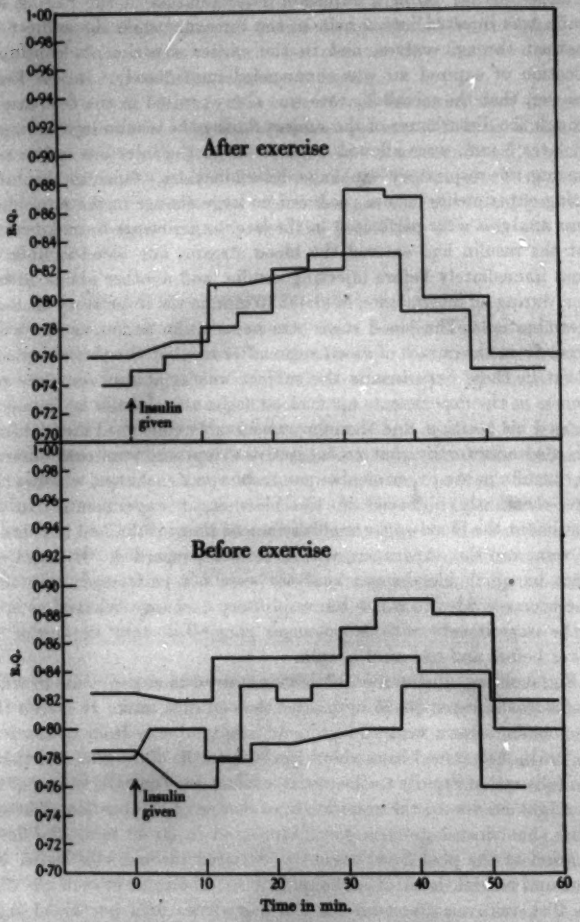

Fig. 6. Douglas, post-absorptive. Effect upon R.Q. of injection of 3 units of insulin. Above, after walking 10 miles at 4-5 miles per hr. Below, before walking.

it falls at first. In all, however, there is a gradual rise, never as great as in the experiments after a walk, and in two curves there is then a very abrupt fall.

The rise of quotient was produced by a considerable rise in CO_2 production, accompanied usually by a smaller rise in O_2 consumption, as Table II shows.

TABLE II. Maximum level of respiratory exchange induced by intravenous injection of 3 units of insulin

Nature of experiment	Initial gaseous exchange c.c. per min.		Gaseous exchange when R.Q. is at its maximum c.c. per min.	
	CO_2	O_2	CO_2	O_2
Before walk	192	246	209	245
	177	225	197	237
	177	215	203	227
After walk	178	241	212	256
	170	224	203	232

To satisfy ourselves that the R.Q. after insulin was a genuine metabolic quotient, and not due for example to hyperpnœa induced by the injection, we have performed experiments in which the alveolar air of Douglas was determined before and after the intravenous injection of insulin. Each record of the alveolar air is the average of the results obtained upon six samples, three inspiratory and three expiratory. Samples of blood for estimation of sugar were taken before and after giving the insulin, to be certain that it had entered a vein. The results obtained are shown in Table III.

TABLE III. Effect of 3 units of insulin, injected intravenously, upon alveolar air and blood sugar of Douglas

Before insulin:	Before walk	After walk	
Alveolar CO_2	38·5 mm. Hg	39·7 mm. Hg	37·7 mm. Hg
Blood sugar	95 mg. p.c.	96 mg. p.c.	96 mg. p.c.
Time after insulin	18 min.	18 min.	16½ min.
Blood sugar	54 mg. p.c.	64 mg. p.c.	57 mg. p.c.
Time after insulin	24 min.	26 min.	28 min.
1st alveolar CO_2 sample	37·0 mm. Hg	37·9 mm. Hg	36·0 mm. Hg
Time after insulin	36 min.	40 min.	39 min.
2nd alveolar CO_2 sample	38·5 mm. Hg	38·9 mm. Hg	38·4 mm. Hg

It will be seen that there is a fall in alveolar CO_2 pressure, which is at its lowest after 20–30 min.; the fall is not large; and comparison with the graphs in Fig. 6 shows that the highest value of the R.Q. is found when the alveolar CO_2 pressure is returning or has returned to normal,

and when there is therefore, if anything, a slight retention of CO_2. The rise of R.Q. after insulin is thus not due to a fall in alveolar CO_2 pressure, and is presumably due to a genuine change in metabolism.

Discussion and conclusions

The present paper and the earlier one of Courtice & Douglas [1936] show that after a period of exercise, when sufficient time has elapsed for the disappearance of any immediate effects such as vascular changes, certain subjects show a series of changes from their condition before the exercise.

The only obvious cause for the low R.Q. after exercise is, as Courtice & Douglas suggest, a depletion of the carbohydrate stores of the body, and the fact that Mills only showed such a depression very inconstantly is confirmatory evidence for this suggestion, as his diet was rich in carbohydrate. The possibility that the immediate cause of the low R.Q. is conversion of fat to carbohydrate has been discussed by Courtice & Douglas, and there is not much to add to their remarks. If we can apply to man the results obtained on animals by hepatectomy, which indicate that the liver is continuously producing considerable amounts of glucose even when the R.Q. is low, and if we can also apply the results obtained on cats by Tsai & Yi [1934, 1936] by the angiostomy technique, which indicate that during the day the liver secretes considerably more carbohydrate than it takes up, then the conclusion is inevitable that new carbohydrate must be formed by the liver; but since we never found a quotient below 0·71, any gluconeogenesis never did more than keep pace with the rate of carbohydrate consumption. Brand & Krogh [1935] have, however, performed experiments upon rats in which an hour after a long spell of work the R.Q. was 0·65, indicating that gluconeogenesis was proceeding fast enough to replenish the glycogen stores; and they found indeed that though after the exercise the glycogen content of liver and muscles was severely depleted, there was a distinct rise in their glycogen content after subsequent rest.

It is possible that in the depression of the R.Q. two factors are involved, one a decrease of carbohydrate combustion in certain tissues, producing a fall in the R.Q. without ketosis, and the other a gluconeogenesis from fatty acids which, according to F. G. Young [1936], is always occurring when there is ketosis.

The depression of the glucose tolerance after exercise had already been observed by Staub [1921]. Our observations strongly suggest that

it is connected with depleted carbohydrate stores, for when these stores are abnormally full, as in Mills at all times and in Douglas on a high carbohydrate diet, exercise does not depress the tolerance. We are familiar with other states in which low R.Q. and low glucose tolerance are associated, such as starvation and living on a diet poor in carbohydrate, but in these the low tolerance is due to low sensitivity to insulin, which does not seem to be the case in our experiments. It may be that the normal disposal of excess glucose is not entirely due to insulin, and that changes in glucose tolerance can occur with a normal secretion and activity of insulin; indeed Soskin *et al.* [1936] have argued that the form of the glucose tolerance curve is unconnected with the action of insulin.

The delay of recovery from insulin hypoglycæmia which we observed after a walk is not readily correlated with any of the other phenomena, except perhaps the greater elevation of the R.Q. by insulin after a walk; it is similar however to that observed by Goldblatt [1936] in thyroidectomized, and by Cope & Marks [1934] in hypophysectomized, animals, and so may indicate some endocrine readjustment.

Ketosis seems, as Courtice & Douglas observed, to coincide with a low R.Q., except for our observation that if ketosis is produced by a walk, and abolished by intravenous injection of insulin, it has not reappeared 2 hr. later, although the R.Q. has fallen to its former low level in under an hour.

Summary

The metabolism of two subjects, who were not in athletic training, has been studied before and after a walk of 10 miles in the post-absorptive state.

One subject, Mills, who lived habitually upon a high carbohydrate diet, showed an inconstant lowering of the R.Q. after the walk, but no ketonuria nor depression of the glucose tolerance, even when he restricted the carbohydrate in his diet.

The other subject, Douglas, who habitually consumed a lower proportion of carbohydrate, always showed a low R.Q. and ketosis after a walk unless he greatly increased the carbohydrate in his diet. His glucose tolerance was considerably depressed after the walk, but if he had been living upon a high carbohydrate diet this depression was absent. The R.Q. after glucose was also determined.

Curves of blood sugar have been plotted for Douglas after intravenous injection of insulin. Those after exercise did not differ from those before in the phase of falling blood sugar, showing that the depressed glucose tolerance was not due to reduced sensitivity to insulin. After

exercise there was, however, a delay in the return of the blood sugar to normal, and determinations of respiratory exchange after injection of insulin suggested that this may have been due to a higher level of carbohydrate metabolism at this period. Insulin was found to raise the R.Q. much farther after exercise than before.

The possible bearing of these phenomena upon the regulation of carbohydrate metabolism is discussed.

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THE ACTION OF ADRENALINE IN ACCELERATING THE REMOVAL OF THE BLOOD SUGAR BY THE PERIPHERAL TISSUES

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(*Received* 16 *May* 1938)

After exclusion of the liver from the circulation in the previously intact unanæsthetized rabbit, the blood sugar declines until death occurs in hypoglycæmia. It was observed that the course of this decline is not smooth but interrupted by waves [Himsworth, 1938]. The mechanism of these periodic interruptions of the fall of the blood sugar was not clear but, as exclusion of the liver eliminated the central source of blood sugar, it was difficult to explain them save as the result of inflow of sugar from a hitherto unsuspected source of blood glucose in the peripheral tissues. The present work was undertaken in an attempt to localize this source.

It seemed reasonable to suppose that if this peripheral source were in the nature of a store of glucose, then glucose would be liberated from it in response to the same agents which evoke outpouring of glucose from the main central store in the liver. The most convenient agent for this latter purpose is adrenaline. The influence of adrenaline upon the blood sugar of dogs which had been recently hepatectomized under general anæsthesia has been tested by Mann [1927]. He found no effect, but his experiments are open to criticism on two points. First, he did not measure the blood-sugar level sufficiently often to exclude any but gross effects, and secondly, recent trauma and recent anæsthesia continue to affect the carbohydrate metabolism of the animal for a considerable time after its apparent recovery [Himsworth, 1938]. For this reason it was determined to investigate the effect of adrenaline on unanæsthetized rabbits from which the liver had been instantaneously and painlessly excluded, and from which in consequence the complicating effects of trauma and anæsthesia were absent. Under these conditions

clear evidence was obtained of a fall in blood sugar after the adrenaline injections.

In the intact animal the hyperglycæmia following injection of adrenaline is well known, and it has long been securely established that this effect can largely be attributed to acceleration of hepatic glycogenolysis. But as more quantitative studies of this phenomenon were made, the view arose that this sustained hyperglycæmia could not be accounted for simply by an outpouring of endogenous sugar into the blood unless there were also at the same time a retardation of the peripheral utilization of blood glucose. The arguments in favour of this view have been recapitulated in a review by Cori [1931], and can be summarized as follows. First, the amount of glucose it is necessary to infuse into an intact animal in order to produce a degree of hyperglycæmia comparable to that following injection of adrenaline is considerably greater than the total amount of glucose stored previously as glycogen plus that newly formed, during the experiment, from protein. Therefore, either adrenaline stimulates formation of glucose from fat, or after its injection the rate of glucose utilization is reduced. The former alternative being considered untenable, the latter alternative was suggested. Secondly, hyperglycæmia following ingestion of glucose is characterized by an increase in the difference between the blood-sugar level in arterial and venous blood, whilst adrenaline hyperglycæmia, in contrast, results in little or no increase of this difference [Wiechmann, 1927; Cori & Cori, 1929*a*, *b*]. A peripheral source of blood sugar not having been detected, these findings were interpreted as due to adrenaline inhibiting the peripheral utilization of glucose. Some difficulty was experienced in reconciling this explanation with the well-established fact of the calorigenic action of adrenaline, but this was met by postulating that adrenaline stimulated the utilization of fat and protein whilst inhibiting the utilization of carbohydrate.

Although this conception of the inhibitory action of adrenaline offers one explanation of its effects on carbohydrate metabolism, it is at variance with what we know of adrenaline action in general. The general action of adrenaline has been summarized by Cannon [1915] in the "emergency theory", a theory which postulates that its effect is to facilitate rapid and efficient action of the body. But if adrenaline inhibits the utilization by the muscles of the carbohydrate on which they normally rely for their source of energy, its action would be the reverse of what would have been expected. The experiments recorded in this paper, by demonstrating directly that adrenaline accelerates the removal

of blood sugar by the peripheral tissues, remove this discrepancy, and permit a conception of its action on carbohydrate metabolism which is in more accord with what is known of its role in the rest of the body.

Methods

Blood sugar. This was estimated in 0·1 c.c. of whole blood by the Hagedorn-Jensen method. Blood was drawn direct from a puncture in the ear vein. It has been shown previously that the non-fermentable, reducing substance in rabbit's blood, as estimated by the Hagedorn-Jensen method, varies only within narrow limits. In sixty-three separate samples from thirty-five different rabbits the value was 14·63 mg./100 c.c. (S.D. ±5·04) [Himsworth & Scott, 1938]. We have, therefore, thought it permissible to subtract this value from the figures given by the Hagedorn-Jensen method in order to obtain the true blood-glucose values of each sample. When this value has not been subtracted the term blood sugar will be used.

It is now well established that the higher the initial level of the blood sugar the greater the absolute fall at definite times after injection of insulin [Hemmingsen & Marks, 1932; Scott & Dotti, 1932; Himsworth, 1934]. The same rule appears to hold for the spontaneous fall of the blood glucose after exclusion of the liver. In 1932 Scott & Dotti showed that, after injection of insulin into a series of rabbits, uniformity in the insulin depression curves could be demonstrated if the blood-sugar values were expressed not in absolute amounts but as percentages of the initial blood-sugar level. This method has been adopted for comparison of the blood-glucose fall after exclusion of the liver, although the initial blood-glucose levels in this series of experiments, being all close to each other, comparatively little difference in the figures has resulted.

Hæmatocrit measurements. These were very kindly carried out for us by Dr M. Maizels of the Clinical Pathology Department, University College Hospital. Heparinized blood was used.

Adrenaline. A freshly made solution of adrenaline hydrochloride 1/1000 was used. When quantities less than 0·25 mg. adrenaline were to be injected, the above solution was diluted with normal saline five times, immediately before each injection. The injections were given intravenously and slowly, at least 1 min. being taken over the process.

Animals. Male rabbits approximately 2 kg. in weight were used. They were given a daily diet containing about 150 g. of a mixture of one part of oats to two parts of bran and 150 g. of fresh cabbage.

Exclusion of the liver. The action of adrenaline in the peripheral tissues was studied on unanæsthetized rabbits from whose circulation the liver had been excluded. The details of this preparation have been described elsewhere [Himsworth, 1938]. The principle is as follows. At a preliminary operation the portal vein is constricted, and the tributaries above this constriction ligatured so that the pressure is raised in the portal circulation, and in the course of 3 weeks an adequate collateral circulation develops. A second operation is then performed and two loosely tied ligatures put in place, one round the afferent and one round the efferent vessels of the liver. The ends of the ligatures are brought out of the lateral abdominal wall, tied behind the animal's back, and the abdomen closed. Four days later the liver can be instantaneously and painlessly excluded from the circulation in the unanæsthetized animal by tightening the ligatures. By this method the animal is given time to recover from the operative procedures, and the complicating factors of trauma, fright and anæsthesia are absent at the time when the experimental observations are made.

In the 4-day interval between the second operation and the test, the animals were kept in a warm room, given 40 c.c. of 10 p.c. glucose in water subcutaneously each night, and received, in addition to their ordinary diet, a solution of 20 p.c. sucrose in water to drink.

Results

Intact rabbits. The injection of adrenaline intravenously causes a pronounced hyperglycæmia. In Fig. 1 are shown two experiments on the same animal, one after injection of 0·025 mg., and the other, performed 2 days later, after injection of 0·25 mg. of adrenaline. With the smaller dose the maximum degree of hyperglycæmia is attained 10 min. after the injection, and the blood sugar commences to fall in 15 min. With the larger dose the blood sugar rises much higher and the maximum hyperglycæmia is not reached until 40 min. after the injection.

Rabbits in which the liver was excluded. In Table I are shown the data from two groups of rabbits, in which the liver was excluded, showing that the two groups of animals were comparable. In one group the blood glucose was allowed to decline spontaneously until death occurred from hypoglycæmia. In the other group, 21 min. after exclusion of the liver, 0·25 mg. of adrenaline was injected intravenously. In Table II figures showing the rate of fall of the blood glucose in each group of animals are given. It will be seen that the standard deviation of the blood-glucose values at definite times after exclusion of the liver are sufficiently

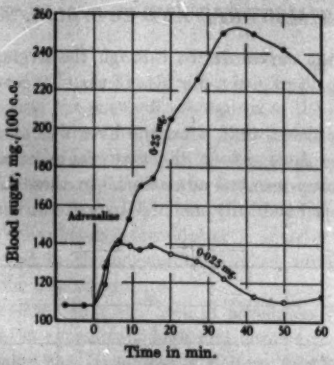

Fig. 1. Two experiments performed on the same rabbit, in the fasting state, at an interval of 2 days. The first (circles) shows the degree of hyperglycæmia produced by injecting 0·025 mg. of adrenaline intravenously; the second (disks) the effect of 0·25 mg.

TABLE I. Rabbits in which the liver was completely excluded

	Controls	Adrenaline injected
No. of animals	7	7
Wt. of animals (kg.)	2·030 (0·220)	2·060 (0·210)
Liver wt./body wt.	0·045 (0·011)	0·050 (0·005)
Time of first convulsion (min.)	278·5 (68·7)	205·7 (36·7)
Time of death (min.)	291·8 (70·0)	209·3 (39·0)
Initial blood glucose (mg./100 c.c.)	101·0 (7·0)	104·5 (12·84)

The figures in brackets show the standard deviation.

TABLE II. Rabbits in which the liver was completely excluded

Spontaneous fall			Adrenaline injected		
Time in min.	Fall of blood glucose	S.D.	Time in min.	Fall of blood glucose	S.D.
0	100·0	0·0	0	100·0	0·0
5	89·0	1·6	5	90·3	3·1
10	83·0	4·7	10	85·6	7·2
15	79·6	5·9	15	80·3	4·1
20	75·4	7·6	20	74·1	5·8
—	—	—	21–22½	0·25 mg. adrenaline intravenously	
—	—	—	25	63·3	4·4
30	76·4	7·1	30	63·3	5·1
—	—	—	35	62·3	12·0
40	71·9	4·6	40	52·7	10·7
50	64·6	8·7	50	51·3	11·2
60	59·0	7·7	60	46·3	10·2
70	53·6	9·0	70	46·6	10·9
80	52·7	7·6	80	42·7	6·0
90	51·1	8·4	90	37·0	9·1
105	50·9	6·2	105	40·4	7·1
120	50·9	10·1	120	37·3	13·1
135	43·3	8·9	135	34·8	8·8
150	40·9	7·9	150	43·0	12·3

S.D. = Standard deviation.

small to permit the curves drawn through the average values of the blood glucose (Fig. 2) to give a fair idea of the changes in the two series of experiments.

In Fig. 2 it is shown that, after the liver was excluded, the blood glucose fell at the same rate in the two groups of animals until the animals of one group received adrenaline. In these animals the blood glucose then dropped suddenly, remained steady at the new level for a

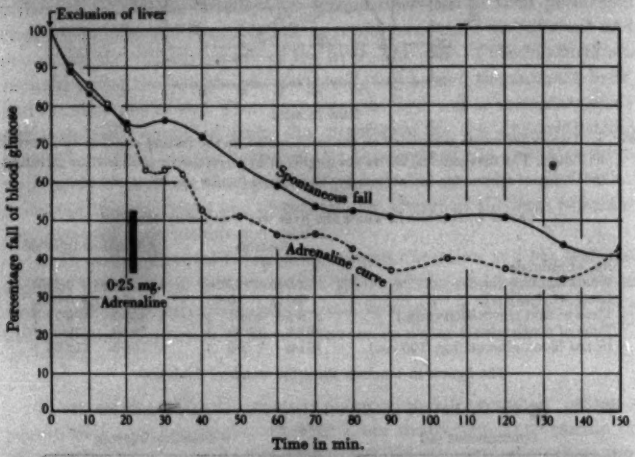

Fig. 2. Comparison of the average rate of fall of the blood glucose, expressed as a percentage of the resting value, in two groups each of seven rabbits. (1) After complete exclusion of the liver (disks), and (2) after complete exclusion of the liver followed 20 min. later by intravenous injection of 0·25 mg. of adrenaline (circles).

few minutes, then commenced to decline, keeping at a lower level than the control until towards the end of 2 hours the curves of the two converge. It is noteworthy that the spontaneous decline curve in the control animals lies, save at one point, outside the range of the standard deviations of the blood-glucose values for the curve after injection of adrenaline until the two curves converge 2 hours later. This result demonstrates clearly that after injection of adrenaline the rate of removal of blood glucose by the peripheral tissues is increased. Supporting evidence is provided by the times of first convulsion and of death in these animals. From Table I it will be seen that the animals injected

with adrenaline convulsed and died about an hour and a quarter earlier than did the control animals. We attach, however, much less significance to the convulsion and the survival times than to the rates of fall of the blood sugar. Nevertheless, one point is shown clearly by these times, and that is that in the adrenaline-injected animals, in contrast to the controls, death followed rapidly on the first convulsion. In the actual experiments this difference early attracted attention. In the control animals several convulsions spread over a quarter or half an hour preceded death, but in the adrenaline-injected animals death usually occurred during the first convulsion.

It may be objected that the dose of adrenaline (0·25 mg.) used in these experiments is excessively high and outside physiological limits. The reason for using this large dose is that we wished to establish unequivocally the phenomena following adrenaline injection. We have, however, been able to demonstrate a smaller but definite effect with 0·025 mg. With such small doses the effect lasts only about 15 min. (cf. Fig. 1). It is also worthy of note that this effect can be demonstrated repeatedly after each of a series of injections on the same animal.

Whilst the curve drawn through the average values of the blood glucose (Fig. 2) establishes the constancy of the depression of the blood glucose resulting from injection of adrenaline it necessarily, by the nature of the process of obtaining averages, smooths out the variations obtained on individual rabbits. In Figs. 3*a* and *b* are shown typical results from individual experiments of injecting 0·25 mg. of adrenaline 20 and 120 min. after exclusion of the liver. These curves illustrate well two striking features of the blood glucose after injection of adrenaline. First, the rapid rate of fall of the blood glucose; secondly, the rise of blood glucose which quickly follows this fall. The rapid fall of blood glucose is a constant finding; the recovery phase is seen in practically every case.

The degree of recovery and the rapidity of its occurrence after adrenaline injection appears to depend upon the time after exclusion of the liver that the adrenaline is injected. When the injection is made 20 min. after exclusion (Fig. 3*a*), recovery commences in about 5 min. and the blood glucose rapidly rises to a level not far short of that which it would have reached if adrenaline had not been injected. When the injection is made 120 min. after exclusion (Fig. 3*b*) recovery does not occur until 20 or 30 min. after injection. At later times still recovery may fail to occur, and adrenaline then produces in the space of 10 or 15 min. vigorous and fatal hypoglycæmic convulsions. We have no

doubt that this latter dramatic result is attributable to the injection of adrenaline, for by this means we have quickly produced fatal hypoglycæmic convulsions in rabbits showing no signs of the ataxia which normally is present in the half hour before spontaneous hypoglycæmic convulsions appear, and in which the blood-glucose level was still

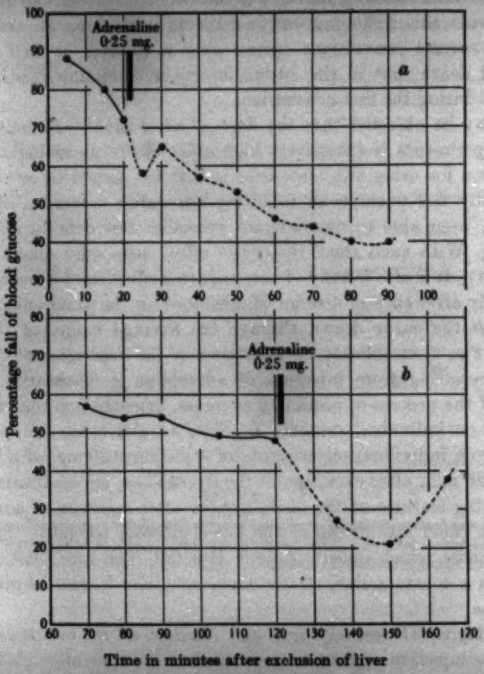

Fig. 3. *a*, Curve showing the effect in an individual experiment of injecting intravenously 0·25 mg. of adrenaline 21 min. after exclusion of the liver. *b*, Curve showing the effect in an individual experiment of injecting intravenously 0·25 mg. of adrenaline 120 min. after exclusion of the liver.

sufficiently high for survival of a further 1 or 2 hours to be expected. The recovery of the blood glucose seen after earlier injections is, however, not permanent, for it is followed by a second slower but enduring fall. It is to this second fall that the persistence of the blood glucose at a

lower level in the curve after adrenaline injection than in the spontaneous decline curve is to be attributed (Fig. 2).

The rate of fall of the blood glucose after injection of adrenaline shows two points of interest, its speed and its depth. The speed of fall is remarkable in being much faster than that produced in these preparations by injection of insulin intravenously, and further in being independent of the initial blood-glucose level. It has been mentioned previously that the higher the initial blood-glucose level, the faster the rate of and the greater the degree of depression after insulin injection. After injection of 0·25 mg. adrenaline the same rapid rate of fall is seen whether the injection be made 20 min. after exclusion when the blood glucose is high (Fig. 3*a*) or 120 min. after exclusion when the blood glucose is relatively low (Fig. 3*b*). The depth of fall is related to the rapidity with which the recovery phase occurs; the sooner this appears the more quickly the fall is curtailed.

In view of the above findings, it was thought advisable to investigate whether the changes observed could be explained as due to rapid changes in hæmoconcentration. Hæmatocrit determinations were made before exclusion of the liver, immediately before injection of 0·25 mg. of adrenaline (17 min.), 5 min. after injection (26 min.) and 70 min. after

TABLE III. Hæmatocrit reading (red blood cell vol. p.c.)

No. of animal	Before exclusion of liver	15–16 min. after exclusion	Adrenaline 0·25 mg. 21–22 min.	25–26 min. after exclusion	91–92 min. after exclusion
D.H. 146	30·5	34·5	—	34·8	30·3
D.H. 147	34·2	35·3	—	38·1	35·4
D.H. 148	33·0	34·5	—	34·9	35·0

Each figure is the average of two duplicate estimations.

injection. The results, shown in Table III, indicate that the effect of adrenaline on the hæmoconcentration is negligible. A slight concentration of the blood follows hepatectomy, but the reason for this is not clear.

DISCUSSION

The interpretation of the phenomena following the injection of adrenaline can be conveniently discussed in two parts: first the significance of the fall, recovery and subsequent fall of blood glucose which immediately follows the injection; and secondly, the gross effect of adrenaline in accelerating the disposal of blood glucose in the peripheral tissues.

There are two alternative simple explanations of the fall of blood glucose following injections of adrenaline. It may result from a facilitation of the transference of glucose between blood and peripheral tissues, in which case the removal may be only transitory; or it may result from increased utilization of the blood glucose by the peripheral tissues, in which case the removal of glucose from the blood is permanent. According to which explanation is favoured the interpretation of the subsequent phenomena must differ. In the light of the first explanation, transference being facilitated in both directions, the fall of blood glucose is due to facilitation of transference out of the blood, whilst the recovery phase may be due to "diffusion" back of this same glucose. Such an explanation would accord with the hypothesis developed by Soula [1933] that after ingestion of glucose a considerable storage of sugar occurs in the periphery, and that as the alimentary hyperglycæmia falls this sugar diffuses back into the blood. The explanation of the second fall on this basis is, however, difficult, and for this reason and also because the second explanation accords better with our other observations on the blood glucose, we prefer to regard adrenaline as causing the permanent transference of sugar from the blood to the peripheral tissues.

On the basis of this second explanation, whilst increased utilization of blood glucose explains the fall, the recovery must be explained by the inflow of glucose into the blood, at a rate sufficient to mask the increased utilization, from some unknown source. The secondary fall would then be explained as the resultant of a continuance of the increased rate of utilization and a waning of the inflow of glucose. As there is no known peripheral source of blood glucose, the explanation is offered with diffidence, but from the following examples it can be seen that there are several observations which can be explained on this basis. Weichmann [1927], Cori & Cori [1929*a*, *b*] have shown that, in adrenaline hyperglycæmia, the difference between the sugar content of arterial and of venous blood is not as great as in a comparable hyperglycæmia induced by ingestion of glucose. As our experiments show that adrenaline increases the uptake of glucose by the peripheral tissues, the results of these workers appear to be most easily explained as due to a masking of the increased peripheral utilization by the inflow into the venous blood of glucose from some peripheral source. Again not infrequently in pathological states, such as diabetes mellitus, venous blood-sugar values higher than those in arterial blood have been reported. One of the pieces of evidence adduced by Cori [1931] in support of the hypothesis that adrenaline inhibits peripheral sugar utilization, is that to

produce hyperglycæmia of the degree and duration seen after injection of adrenaline, requires more sugar than could be provided by breakdown of all the liver glycogen plus the glucose newly formed from protein. This discrepancy might be, at least in part, removed if to these two sources of glucose were added the glucose from a peripheral store. Lastly, it is difficult to see any explanation of the periodic waves which interrupt the curve of spontaneous blood-sugar fall after exclusion of the liver, other than the inflow of glucose from some peripheral source. We, therefore, consider it possible that there is a source of available glucose in the peripheral tissues and, if this is conceded, the view that adrenaline stimulates the peripheral utilization of blood sugar becomes acceptable.

With regard to the mechanism by which glucose is mobilized from this peripheral source, one observation alone appears fairly certain. In an unanæsthetized rabbit *in good condition*, from which the liver has been excluded, a rapid drop of blood glucose, from any cause, is usually followed by a rise of blood glucose. This present work was commenced under a misconception. It was felt that the important part of the waves interrupting the spontaneous fall was the rise. On reviewing our previous results it became clear that a rise of blood glucose had always been preceded by a sudden fall. This is true whether the fall occurs spontaneously or after injection of adrenaline or, in some instances, after injection of insulin. It should be emphasized that to demonstrate this rise the animal must be in good condition, have had no anæsthetic for at least 3 days previously, and have ample stores of carbohydrate available in its body. If, before exclusion of the liver, the animal is in bad condition or its carbohydrate stores are depleted, the fall of blood sugar is uninterrupted and rapid [Himsworth, 1938]. To guard against such depletion 40 c.c. of 10 p.c. glucose in saline were injected each evening for the 4 days before the test. It has been pointed out in this paper that the time after adrenaline injection at which the recovery phase appears is longer the later after exclusion of the liver that adrenaline is injected, until finally a stage is reached at which no recovery occurs. This retardation and weakening of the recovery phase suggest a progressive diminishing of the carbohydrate content of the peripheral store, and it is easy to explain on similar lines why in an animal in which after operation this store has not been replenished by liberal injections of glucose, the spontaneous fall of blood sugar may be more rapid than in the case of the animal whose good condition has thus been secured. If this consideration is correct, then it follows that the inflow of glucose from a peripheral source, even in the absence of its inflow being stimu-

lated by sudden falls of blood glucose, plays a very considerable part in supporting the blood-glucose level after exclusion of the liver, and thus in prolonging the life of the animal.

Consideration of the gross effect of adrenaline in accelerating the disposal of glucose by the peripheral tissues has been largely included in the above discussion, but one further point requires explanation. Why, after the blood glucose has been lowered by adrenaline (Fig. 2), does the curve subsequently tend to converge with the control spontaneous decline curve? On inspection of Fig. 1 it will be seen that the peak of hyperglycæmia, after injection of 0·25 mg. of adrenaline into the intact animal, is reached in 35 min. This waning of adrenaline hyperglycæmia may result either from weakening of adrenaline action or from exhaustion of the stores of liver glycogen, or from both these factors acting together. Assuming that the main force of adrenaline action is spent 40 min. after the injection of 0·25 mg., the adrenaline curve in Fig. 2 can be considered from 60 min. onwards as an ordinary spontaneous decline curve. At 60 min. it has fallen to 46 p.c. of its original value, whilst the spontaneous curve has only fallen to 50 p.c. As the spontaneous decline of the blood sugar follows roughly a hyperbolic curve, the subsequent fall of the adrenaline curve will be slower than that of the control spontaneous decline curve and the two curves will naturally tend to converge.

The original object of this present investigation was to discover the cause of the waves which occur in the falling blood-glucose curve after the liver has been excluded from the circulation. The foregoing results allow a tentative explanation of this phenomenon.

Cannon *et al.* [1924] have shown that hypoglycæmia stimulates the secretion of adrenaline and this, in the intact animal, by liberating glucose from the glycogen in the liver brings about a return of the blood glucose towards normal levels. With the return of the blood glucose adrenaline secretion ceases. This secretion of adrenaline is not delayed until profound hypoglycæmic levels are reached, but commences when the blood sugar has fallen only as far as 70 or 80 mg./100 c.c. As a fall of this magnitude occurs in the first 15–20 min. after exclusion of the liver, it is probable that at this time the adrenal glands are stimulated to secrete adrenaline. The sequence of events would then be as follows. The adrenaline secreted would cause a fall of blood glucose, and this fall would be followed by a rise. With the subsequent fall further secretion of adrenaline would be evoked and the sequence of fall and recovery repeated. The result would be that the curve of decline of the blood glucose would be interrupted by a series of waves. This suggestion could

be put to the test on rabbits in which the liver is excluded after removal of both suprarenal glands. Unfortunately we have been unable to produce such preparations as the necessary operations, whilst not technically difficult, resulted in the death of the animal within 24 hours of the final operation for exclusion of the liver.

Summary

1. The effect of injecting adrenaline intravenously into unanæsthetized rabbits from whose circulation the liver has been excluded, has been investigated.

2. Adrenaline, in the doses employed, accelerates the rate of removal of glucose from the blood by the peripheral tissues.

3. It is suggested that the accelerated removal of the blood glucose results from increased utilization of this sugar by the tissues under the influence of adrenaline.

4. Further evidence is presented which suggests that there exists in the extrahepatic tissues a store of carbohydrate which can supply glucose to the blood.

5. The waves which interrupt the spontaneous fall of the blood glucose after exclusion of the liver from the circulation may be attributable to the peripheral effect of adrenaline secreted in response to the falling blood glucose.

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J. Physiol. (1938) 93, 173–193 612.817

ON THE MECHANISM OF INHIBITION AND EXCITATION OF CRAYFISH MUSCLE[1]

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(*Received* 7 *September* 1937)

The nerve-muscle preparation of the crustacean claw has long served as an example of peripheral inhibition [Biedermann, 1887; Hoffman, 1914; and others], and the explanation of this inhibition has excited much experiment and discussion. A summary of this earlier work is contained in the paper of Wiersma & van Harreveld [1934], and strong evidence for the existence of purely inhibiting fibres is pointed out. Since this latter publication the individual motor and inhibiting fibres have been isolated, and stimulation of these fibres has demonstrated their respective functions [van Harreveld & Wiersma, 1937].

To date very little has been found that will elucidate the intimate nature of this peripheral inhibition. Wiersma & van Harreveld [1935] and Serkoff [1936] have reported the absence of a distinct electrical sign from the muscle when the nerve-producing inhibition was stimulated; Biedermann [1887] and du Buy [1935] found an electro-positivity. Diminution or disappearance of the normal muscle action current during inhibition has been reported by Segaar [1929], Wiersma & van Harreveld [1935], and Serkoff [1936]. None of these authors, however, studied the essential conditions necessary for this effect.

Lack of an effective method of separating and stimulating the nerve fibres innervating a single muscle probably constitutes the main reason that more has not been found out about this system. The application by van Harreveld & Wiersma [1936] of micro-manipulated electrodes has furnished such a method, so that now, in the study of inhibition, the

[1] A preliminary communication of part of the results appeared in *Proc. Nat. Acad. Sci., Wash.*, 22, 502 (1936).

disadvantages inherent in simultaneous excitation of the antagonistic muscle (the tendon of that muscle having been cut), or in destroying that muscle, can be avoided.

Method

The rather surprising nature of some of our results, especially the fact that complete mechanical inhibition of the abductor could be obtained with or *without* reduction of the normal action current of that muscle, impels us to describe our methods in greater detail than would otherwise be necessary.

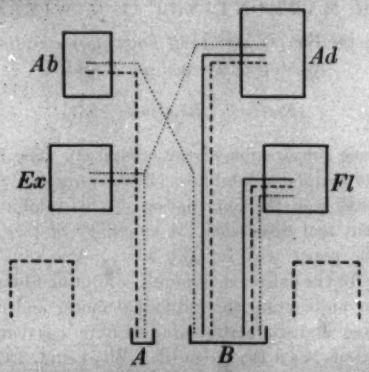


Fig. 1. Scheme of innervation of the four distal muscles of the cheliped. *Ab* represents the abductor muscle; *Ad*, the adductor; *Ex* and *Fl*, the extensor and flexor of the propodite, respectively. The two muscles indicated by broken lines are those lying in the meropodite; they were always removed to allow preparation of the nerve fibres. The bracket *A* indicates the thin nerve bundle, and *B*, the thick bundle. The unbroken lines represent fibres causing a fast contraction; the broken lines, those causing a slow contraction; and the dotted lines, the inhibitory fibres. (After van Harreveld & Wiersma [1937].)

Two species of crayfish, *Cambarus clarkii* and *Astacus trowbridgii*, were used in these experiments. The motor and inhibitory innervation of the muscles of the cheliped of these animals has been worked out by van Harreveld & Wiersma [1937]. Their scheme of innervation for the distal four muscles of the claw is reproduced in Fig. 1. The preparation principally used was that of the abductor muscle of the cheliped and the two axones innervating it. It will be seen from the diagram that one of these fibres excites this muscle to contract in a "slow" fashion, while the other inhibits this contraction. The former fibre, the motor one, also

innervates the extensor of the propodite, but this muscle has a separate inhibiting axone which runs in the same nerve bundle as its motor companion. The inhibitor of the abductor travels, at least in the meropodite region, in another bundle which contains also the two motor axones to the abductor.

The nerve fibres, running in the exposed meropodite region of the claw, were prepared with needles and tested by means of two pairs of micro-manipulated electrodes, the nerves being submerged in van Harreveld's crayfish solution when not being stimulated. The abductor preparation was made by isolating the abductor-extensor motor axone and the extensor inhibitor in one bundle and the abductor inhibitor in another. All other motor axones were discarded. Since it was found that stimulation of sensory fibres had no influence on the results, sometimes a small number of these were left intact in order to afford mechanical support to the prepared fibres. The ease and sureness with which this preparation can be made and the obviating of all doubt as to which fibres are being stimulated constitute its definite advantages. The latter point is of particular importance when action currents are led off.

To record the contractions of the abductor preparation the dactylopodite was connected by a heavy thread to one of three types of lever: a lightly loaded isotonic one for recording on a smoked drum, or either an isometric or an auxotonic lever equipped with a small mirror for recording on photographic paper. The type of device used had no effect on the completeness of inhibition.

Action currents from the abductor were led off by means of a pair of Ag-AgCl electrodes bearing small cotton-wool covered loops at their ends. This ensured good contact with the muscle, without, at the same time, injuring it. The grounded electrode was placed on the surface of the abductor muscle through a small hole pierced in the chitin. The other lead was placed in the gelatinous substance occupying the immovable dactyl of the propodite. A condenser-coupled amplifier and a Matthews oscillograph were used in recording the action currents. A diagram of the way in which this preparation was set up for simultaneous recording of action currents and contraction is shown in Fig. 2.

The mechanical inhibition of other nerve-muscle preparations of these same animals was also studied, and the making of these preparations will be briefly described. The "slow" contraction of the adductor of the cheliped can be inhibited by the axone which also inhibits the extensor of the propodite (see Fig. 1). The inhibiting action of this fibre is much more pronounced on the latter muscle, which was therefore used for the

tests. After the inhibitor had been isolated the two motor fibres to the adductor were prepared, all other motor axones being cut away.

The flexor of the propodite is also fed by three axones, two motor and one inhibitor, which run closely together. Its preparation was made by separating these fibres from one another and from the other motor fibres of the claw. This preparation is a typical example of a triply innervated muscle and is the only one suitable for preparation to be obtained in the crayfish [van Harreveld & Wiersma, 1937].

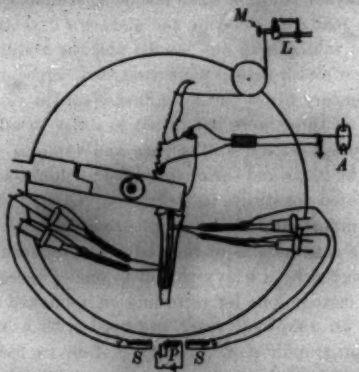


Fig. 2. Set-up for simultaneous recording of the electrogram and mechanogram from the abductor muscle during inhibition. The cheliped is clamped so that the meropodite dips into physiological solution. The inhibitor axone, which is prepared distally in the meropodite as a bridge preparation, runs in the thick bundle indicated to the left. The motor fibre to the abductor lies in the thin bundle. As shown, the stimulation of the two bundles is arranged to give simple inhibition. For other details see text. Outside the circle the figure is schematic. *L* represents an auxotonic lever on which a mirror, *M*, has been mounted. *A* is the first stage of amplification. *P* represents the primary of the inductorium, and *S*, *S* the two secondaries.

Results

(1) *The effect of inhibition on the mechanogram*

The effect of the frequency of inhibition on the completeness of the suppression of the contraction was investigated. Inhibition was complete for the abductor when both fibres were stimulated faradically. Using a Lewis contact breaker for varying the frequency of the induction shocks to the inhibitor, we determined the frequency at which inhibition was "just complete": i.e. the minimum frequency of inhibition necessary

to suppress the contraction when stimulation of both fibres was started simultaneously. This frequency did not suffice to cause a complete relaxation of the muscle when it was given in the midst of a contraction. It was found that inhibition was "just complete" at 20 per sec. when faradic stimulation of the exciter occurred at 50 per sec. Thus the ratio, frequency of inhibition/frequency of excitation ($R=f_i/f_e$) was 0·4. It was of interest to determine how R_e (R for just complete inhibition) varied with f_e. Stimulating the exciter with a thyratron variable frequency generator, which gave rectangularly shaped waves of 0·5 msec. duration, and using the Lewis stimulator for the inhibitor, we could obtain R_e for a range of values of f_e. In this range R_e varied not more than 15 p.c., as will be seen from Table I.

TABLE I. The frequency ratios for "just complete" inhibition of the abductor

Preparation	f_i/sec.	f_e/sec.	R_e
1	25	60	0·41
	15	38	0·39
2	40	100	0·40
	30	80	0·38
	15	34	0·44
3	44	100	0·44
	33	80	0·41
	20	50	0·40
	15	33	0·45
	40	100	0·40

R_e was also determined for the slow and fast contractions of the flexor of the propodite and the slow contraction of the adductor. It appears that this ratio is different for each of these systems. Table II gives these values for one frequency of excitation.

TABLE II. The frequency ratios for "just complete" inhibition of some other muscles of the cheliped

Muscle system	f_i/sec.	f_e/sec.	R_e
Flexor of the propodite "fast"	60	50	1·2
Flexor of the propodite "slow"	35	50	0·7
Adductor "slow"	50	8	6·2

The high value of R_e for the slow contraction of the adductor shows that this muscle is extremely difficult to inhibit; it is only completely inhibited with low frequencies of excitation. With somewhat higher frequencies inhibition has only a partial effect. When this system is stimulated above 20 per sec., even very high frequencies of stimulation of the inhibitor (200 per sec. or more) usually do not produce any visible sign of inhibition. Therefore, R_e has little significance for this case. We

were never able to obtain any inhibition at all of the twitch contraction of this muscle.

Pantin [1936] obtained good inhibition of the adductor of the claw of *Carcinus*. In a recent investigation we have also found that both fast and slow contractions of the adductor in a number of brachyuran decapods are rather easily inhibited [Wiersma & Marmont, 1938].

The differences in R_e for the two types of contraction of the flexor show that the "fast" contraction is more difficult to inhibit than the "slow". Since it is likely that both contractions occur in the same muscle fibres [Wiersma & van Harreveld, 1938] this result is of great interest.

(2) *The effect of inhibition on the electrogram*

The abductor muscle preparation was used in all the experiments described in the remainder of this paper. The action currents of the abductor are of the slow type and resemble in every respect those described for the slow adduction by Wiersma & van Harreveld [1938]. With indirect faradic stimulation (50 per sec.) they grow for about 5 sec. from no appreciable size on the first few shocks, remain almost constant for about 1 min., and then begin to diminish, whilst the contraction may still increase. Upon repetition of a stimulation within a certain time after a preceding one, they show a faster growth than initially. The time interval after the first stimulation during which this latent effect can be obtained varies with the length of that stimulation.

At first the two axones, the exciter and the inhibitor, were stimulated faradically by means of separate induction coils, which were set at right angles to each other. When the inhibitor was stimulated in the midst of a period of excitation, the action currents appeared during inhibition in a random assortment of sizes. The largest was equal in height to that of the uninhibited action currents, the smallest about one-third the height of the latter. Mechanical inhibition was, of course, complete. Such a record is shown in Fig. 3. From the current escape traces, which are marked on Fig. 3, it appears that the inhibitor impulses are set up quite at random in relation to those of the exciter. The arrow in the figure shows that the action current is most reduced when the inhibitor is stimulated shortly before the exciter. Therefore it might be suspected that the time of arrival at the muscle of the inhibitor impulse in relation to that of the exciting one plays a role in determining the action current height.

Acting upon this assumption, a method was developed whereby the inhibitory impulse could be made to arrive at the muscle at any time

within approximately 3 msec. before or after the exciter impulse, using faradic stimulation. One primary but two secondary coils were used. Each secondary was capable of being short-circuited by a switch, and each was connected to its own pair of micromanipulated electrodes. Thus the stimuli given by these two pairs of electrodes were synchronous, and only one current escape trace was produced. Since the conduction velocity in crustacean nerve is of the order of 5 mm. per msec., the relative time of arrival could be conveniently and gradually varied by shifting the inhibitory electrodes proximally or distally along the length of the nerve relative to the exciting electrodes. In the meropodite as much as 15 mm. of both nerve bundles could often be exposed, and so a total

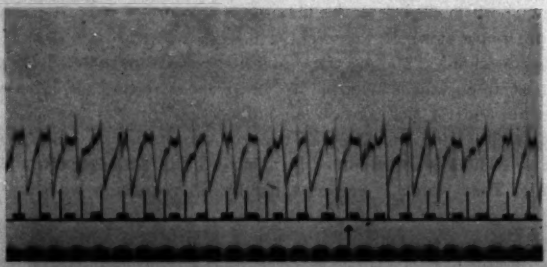


Fig. 3. Action current record taken from the abductor muscle of a preparation in which the inhibitor and exciter axones were stimulated with two asynchronous induction coils. The lines mark the stream escape traces from the inhibiting electrodes, while the blocks represent the latent period of the muscle action current. Time $\frac{1}{5}$ sec.

range of 4–6 msec. could be obtained. Longer (but less exact) intervals were obtained by employing an alternative method which involved setting the two cams of a Lewis contact-breaker at varying angles with one another. Here two complete induction coils were used and the electrodes were placed at the same level on the two nerve bundles. By this arrangement the relative starting times of the exciting and inhibiting impulses could be shifted over a full cycle, but two current escape traces were produced. For these experiments the exciter was stimulated for about 3 sec. before the inhibition was started, a 3 sec. period of simultaneous excitation and inhibition was given, and this was followed by 3 more sec. of excitation alone. If a rest of 3 sec. was intercalated instead of the period of simultaneous excitation and inhibition, the action currents and contraction were not facilitated upon resumption of excitation.

The surmise in regard to the importance of phase was found to be correct. By varying the relative time of arrival, it was possible to obtain a series of "inhibited" action currents, ranging from 20 to 100 p.c. of the normal height. Fig. 4 shows such a series obtained from one preparation. It is especially to be noticed that the mechanical inhibition is complete, regardless of the height of the electrical response. A sensitive auxotonic lever was used in some experiments in order to be certain that mechanical inhibition was always complete. The curves illustrated in Fig. 5 give the relation between the height of the inhibited action current and the time difference for preparations investigated by both methods. For a given preparation the curve found is usually quite constant. Different chelipeds give curves which vary quantitatively from one another, both in regard to the maximum amount of reduction and the time difference at which this occurs.

That mechanical and electrical inhibitions are separable effects seems the most important conclusion to be derived from this experiment. Therefore, and for the sake of convenience and brevity, we shall term "supplemented inhibition" that type of simultaneous inhibition and excitation which is accompanied by a substantial reduction of the action currents. That which is accompanied by no reduction we shall term "simple inhibition".

It was sometimes found that the action currents resulting when the inhibiting impulse were just too late to cause reduction were slightly higher than normal. As this difference was always small and not constantly found, it is still uncertain if it is significant.

Other characteristics pertaining to the height of the electrical response appear in Fig. 4. First, the action currents are reduced somewhat gradually at the onset of the supplemented inhibition but rise immediately (rebound) to their normal height when the inhibiting impulses are stopped; the contraction also rises rapidly to its facilitated height in both cases. In simple inhibition the normal growth (facilitation) of the action currents is apparently the same as though excitation alone were given. Similarly, the reduced action currents during supplemented inhibition assume, upon cessation of inhibition, the same height as those after simple inhibition. Facilitation must be independent of the action current height. This is most clearly shown by other experiments where inhibition and excitation had been started simultaneously, without previous excitation, inhibition being released after about 3 sec.

Fig. 5 shows that in order to obtain sharp supplemented inhibition, the inhibitory impulse must be set up within a total period of 4 msec. about the moment of setting up the exciting impulse. The portion of the

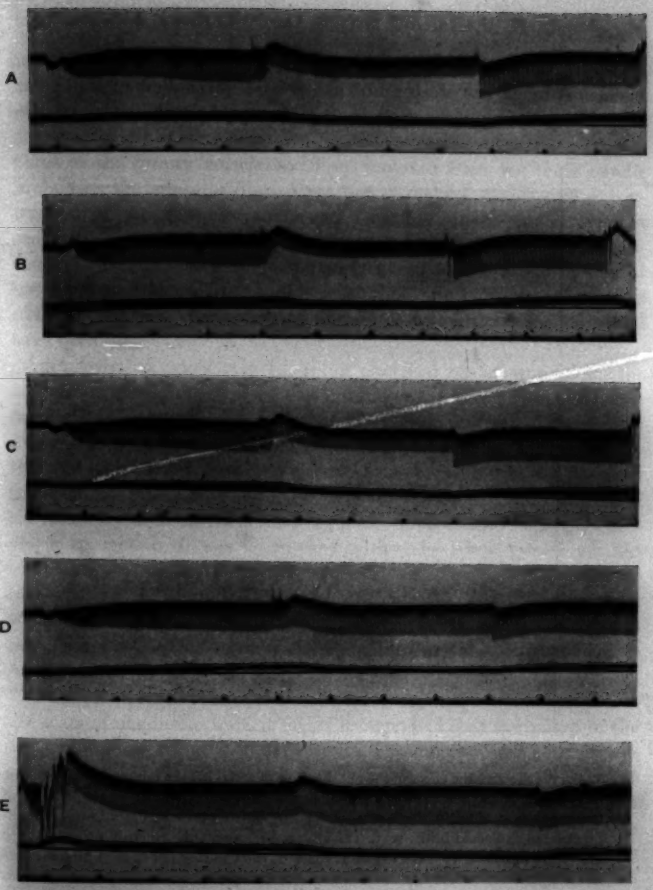


Fig. 4. Action current records and isometric mechanograms from a preparation for which one primary coil only was used for stimulation. The inhibiting and exciting electrodes were connected to separate secondaries, each of which could be short-circuited. In A the inhibiting electrodes were placed 12 mm. distal to the exciting ones in the meropodite; in B the former were 6 mm. distal; in C both pairs of electrodes were at the same level; while in D and E the inhibiting pair were 6 and 12 mm. proximal to the exciting pair, respectively. Inhibition was given during the middle of the period of excitation for 3 or 4 sec. in each case. It will be noticed that mechanical inhibition is always complete. Time 1 sec.

curves to the right of the point corresponding to the minimum action current is much less steep than that portion to the left of this point. Apparently the significance of this is that only if the inhibiting impulse

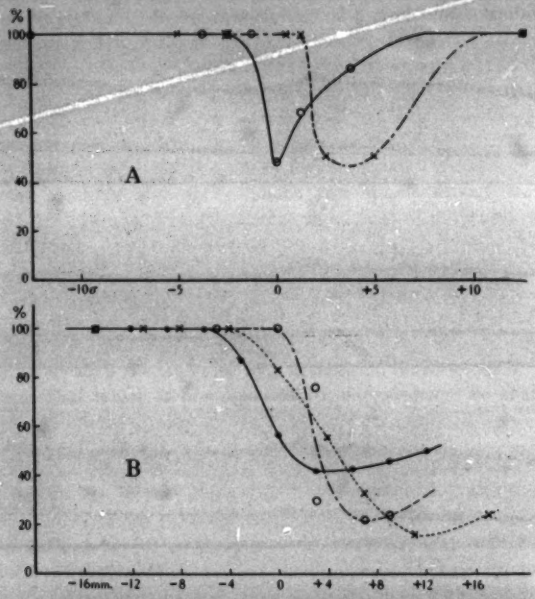


Fig. 5. Curves showing the effect of the relative time of arrival of the inhibiting and exciting impulses upon the height of the action currents. A. Two cases where the starting of the impulses at different times was obtained by shifting the cams of a Lewis contact-breaker, as explained in the text. The abscissæ represent the times in msec. that the inhibiting impulses were started in advance of the exciting ones; the ordinates give the height of the reduced action currents in terms of the normal, uninhibited height. B. Curves for three preparations for which the times were varied by shifting the electrodes in the meropodite. Here the abscissæ represent the amount in mm. that the inhibiting electrodes were distal to the exciting ones, while the ordinates are the same as in A.

arrives at the muscle synchronously with or slightly before the exciting one can reduction of the electrical response occur. It must be remembered that the action currents are led off from a number of muscle fibres.

Before proceeding to analyse these two types of inhibitions further, we shall try to allay any doubts which the reader may have about the reality of this effect. It is certain that stimulation of the adductor did not occur in any of the experiments on the abductor. After the two motor fibres to the former muscle had been removed from the thick bundle containing the inhibitor fibre, there was never any visible sign of contraction of the adductor upon stimulation of that bundle. Such a contraction was always looked for in each preparation. In the experiments on mechanical inhibition described in § 1, a very lightly loaded isotonic lever was used to provide sensitivity. Had a contraction of the adductor occurred, relaxation of the writer below the base-line would have been seen.

Similarly, in every case where an electrical record was taken, the inhibitor was stimulated alone to determine whether an adductor action current was present or not. The method of leading off would easily have detected any electrical sign from this muscle. Even with large amplification and high camera speed, no electrical sign of inhibition was observed (see Fig. 6A). With this method of recording, the form and duration of the reduced action currents resulting from supplemented inhibition were also investigated. It was found that neither the latent period nor the total duration of the electrical response was appreciably changed as compared to the uninhibited action currents (Fig. 6B). These action currents are also monophasic. This monophasicity results, at least in part, from the position of our leading off electrodes, one being on inactive tissue.

When it is recalled that the abductor and the extensor of the propodite are parts of one motor unit, it might be supposed that the action of the latter muscle contributed to our records. This muscle could not contract since its inhibitor runs next to the motor fibre. It was also found that its electrical response was undetectable when abductor action currents were being recorded. This was determined by severing the motor axone at a point between the two muscles, so that the abductor was no longer excited. After this transection no electrical sign was found upon stimulation of the motor fibre (the inhibitors were removed for this experiment).

Finally, the two types of inhibition were always easily obtained from every successfully made preparation. Recently, similar investigations of the same muscle in several marine crustaceans (*Pagurus ochotensis*, *Lozorhynchus grandis*, and *Pachygrapsus crassipes*) showed that supplemented and simple inhibition could be obtained. In *Loxorhynchus*, a spider crab, and *Pachygrapsus*, a shore crab, the abductor inhibitor does not run with the adductor axones, but instead with the abductor fibre [Wiersma & Marmont, 1938]. Therefore the chance of adductor stimulation is even further removed.

A deeper insight into the nature of the two kinds of inhibition was not obtained from experiments where inhibition and excitation were given for short periods. It was clear that electrical inhibition is separable from mechanical inhibition and that facilitation of the action currents

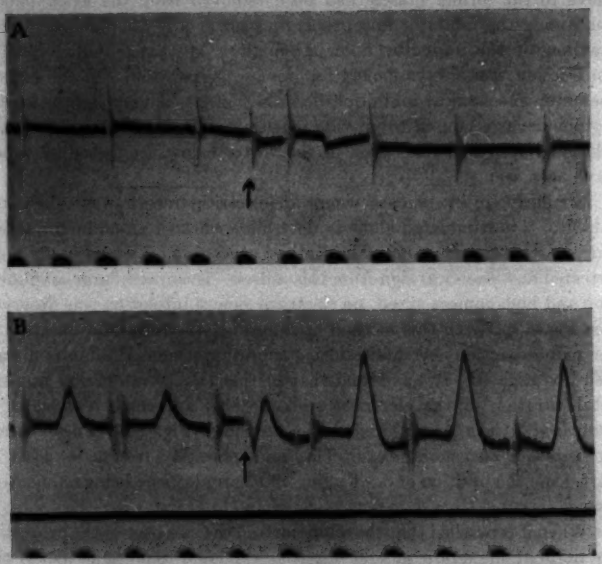


Fig. 6. A. Electrogram of the release of inhibition given alone, taken with a fast camera and high amplification. The arrow shows when inhibition was stopped. The large deflections are the current-escape traces of the break shocks, and the smaller deflections those of the make shocks. Since stimulation of the inhibitor fibre was stopped by short-circuiting the secondary, smaller current-escape traces persist after the point indicated by the arrow. B. Record of the release of supplemented inhibition taken in the same manner. The arrow indicates when the release occurred. It will be seen from the current-escape traces that the latent period remains constant. The same is true for the duration of the action currents. Time $\frac{1}{100}$ sec.

is a process independent of either. That inhibition might be a valuable tool for investigating the nature of peripheral excitation was an interesting prospect. The best use of this tool resulted in experiments where stimulation of the inhibitor and the exciter axones was carried on for long periods. Long simultaneous excitation and inhibition also furnished us

with an excellent proof that the results described in the last section are not artefacts.

Therefore the remainder of the paper will describe the effect of long periods of inhibition alone (§ 3) and of long periods of simultaneous excitation and inhibition (§ 4). In the discussion a scheme of excitation and inhibition of crayfish muscle will be presented.

(3) *The after-effect of inhibition; fatigue of inhibition*

There is an after-effect following a period in which inhibition alone has been given. The after-effect is manifested in an ensuing contraction, which contraction is depressed as compared to a normal or control contraction. Its latent period is lengthened and the rise of the contraction is more concave. Correspondingly, the action currents grow more slowly. In Fig. 7 these effects may be seen.

However, inhibition must be given for a certain minimal period before its after-effect can be demonstrated. When a 3 sec. period of excitation alone was given, as a control, followed by a 3 sec. period of inhibition alone, and this was immediately followed by another 3 sec. period of excitation alone, the two contractions were similar. Only by lengthening the period of inhibition to 1 min. or longer could the second contraction be made to show the depression just described. The time for which this inhibitory after-effect lasts after it has been set up depends not only on the length of the period of inhibition, but also on the freshness of the preparation. A partially fatigued claw will show it for several minutes after an inhibition of 1 to 2 min. duration has been stopped.

The relation between the length of the period of inhibition and the degree of after-effect was investigated, among other ways, by stimulating the inhibitor fibre continuously with the exception of brief intervals during which excitation alone was given in order to test the effect. Fig. 8, reproducing a record from such an experiment, shows that the after-effect reaches a maximum at about 3 min. Test contractions observed after longer periods of inhibition are less depressed, but are not equal to the normal response.

Although a digression, let us report another result of this type of experiment. In a number of preparations faradic stimulation of the inhibitor fibre was still effective after 65 min. or more. The experiment was controlled in the following way: every 5 min. the exciter was stimulated for 3 sec. as a test and during the 3rd sec. inhibition was stopped to test the reality of excitation. This clearly demonstrated the high resistance to fatigue of crustacean nerve, as well as that of the peripheral (mechanical) inhibitory mechanism.

Action currents taken under similar conditions were interesting. Here the inhibitor was not stopped during the test excitation. First, stimula-

tion of the fibres was arranged so that simple inhibition was obtained during the test periods. After 30 sec. of inhibition the test action currents were normal in height, but later they showed a considerable

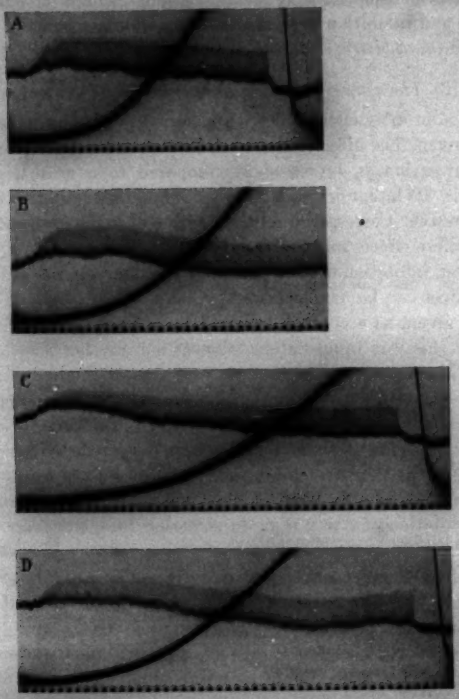


Fig. 7. Electro- and auxotonic mechanogram records showing the after-effect of inhibition Between A and B, and C and D there was a 2 min. rest period, but between B and C the inhibitor axone alone was stimulated for 2 min. The after-effect is shown in C by the depressed contraction and lower action currents, while D shows the degree of recovery attained. Time $\frac{1}{10}$ sec.

reduction which is to be ascribed to the inhibitory after-effect. In test excitations taken still later this reduction was less pronounced, corresponding to the diminishing of the after-effect. When the test excitations were arranged so that supplemented inhibition resulted a rather important

phenomenon occurred: after a certain period of inhibition (10 min. in one preparation), the action currents during the test excitation no longer showed the characteristic reduction of supplemented inhibition. Varying the relative time of arrival at the muscle of the two types of impulses by shifting the stimulating electrodes was ineffective in restoring supplemented inhibition. We must conclude that the mechanism responsible for the action current reduction had fatigued, even though complete inhibition of the mechanical contraction remained for 60 min. in all.

Fig. 8. Records of the course of the inhibition after-effect. The inhibitor fibre was stimulated continuously except for the first 3 sec. of the test periods, the durations of which are shown by the signal marking. The contraction tests (isotonic) were taken at 0, 1, 3, 5, 7, 9, 11, 13 and 15 min. respectively. Note that the contraction at 3 min. is smaller than any of the others. Time 1 sec.

When fatigue of mechanical inhibition does occur it is at first only partial and usually a brief rest is sufficient to give a marked but short recovery. For example, in one partially fatigued preparation, a release of inhibition so short ($\frac{1}{2}$ sec.) that an increase in contraction was not visible, gave, upon renewal, a very marked reduction in the mechanogram which persisted for a short period. Likewise, a brief cessation of inhibition given when only supplemented inhibition had failed, i.e. the action currents were no longer reduced, produced a temporary recovery of the effect.

(4) *The effect of prolonged simultaneous inhibition and excitation*

Upon release of inhibition after short periods (10 sec.) of simple or supplemented inhibition the contractions and action currents were equally facilitated in both cases. When in the same experiment the simultaneous inhibition and excitation was prolonged for a period of 2 min. and the stimulation was arranged for supplemented inhibition, the mechanical response after cessation of inhibition was very greatly facilitated. When the inhibition was simple the rebound was distinctly less (see Fig. 9). The amount of rebound depends only on the length and type of inhibition preceding it. It is independent of the order in which a preparation is treated to the two kinds of inhibition.

Fig. 9. The effects of different periods of supplemented and simple inhibition upon the contractions after release of inhibition. The supplemented inhibition was obtained by placing the inhibiting electrodes 5 mm. distal, the simple by placing them 3 mm. proximal to the exciting ones. The simultaneous inhibition and excitation was given in the period between the two signal markings, which show the duration of the initial and final contractions. Between each record a 3 min. rest was given. In A and B 1 min. supplemented and simple inhibition was given respectively. Note that A is larger than B. In C and D it was simple and supplemented for 2 min., respectively; D is considerably more facilitated than C, but both are larger than after 1 min. In E and F 3 min. of supplemented and simple inhibition were given, respectively. Note that E is somewhat smaller than D, but that F is greatly reduced as compared with C. Time 1 sec.

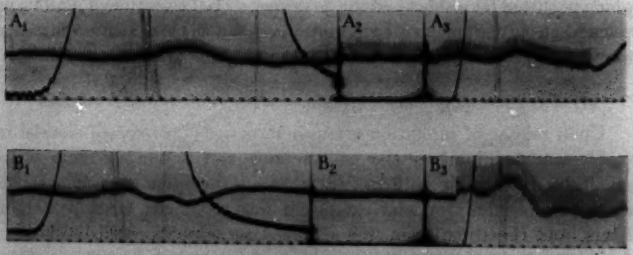


Fig. 10. Record showing the effect of long simple and supplemented inhibition upon facilitation of the action currents. In A_1 simple inhibition was started for a 2 min. period; A_2 was taken after 1 min. and in A_3 the inhibition was released. In B_1 supplemented inhibition was begun; B_2 shows the height of the reduced action currents after 1 min.; and B_3 shows the greatly facilitated height to which they rebounded upon turning off inhibition after 2 min. Time $\frac{1}{10}$ sec.

Nor is this effect limited to the contraction: the action currents also show it. Fig. 10 shows that the maximum action currents obtainable after a period of supplemented inhibition may be almost twice as great as the largest ones taken from the same preparation during or after simple inhibition. As has been stated, the latter follows the same course as the uninhibited action currents. Therefore when the action currents are reduced by inhibition the process responsible for their facilitation is able to exert its effect to far greater extent after release of inhibition. Further, the facilitation of the electrical response probably plays an important part in facilitating the contraction.

This effect of the kind of inhibition on the rebound is important for several reasons. First, it proves that the two kinds of inhibition, simple and supplemented, are not artefacts, for when the inhibition is supplemented *extra* facilitation is manifested afterward. Second, it allows us to draw new conclusions about the process of muscular excitation in Crustacea. These conclusions will be discussed later.

It has been said that the extent of rebound depends on the length and type of inhibition preceding it. The type of inhibition had no effect on the rebound when the duration of inhibition was less than 30 sec.; the contraction and the electrical response were equally facilitated after either supplemented or simple inhibition. The action currents afterwards were the same as if excitation alone had been given for 30 sec. But if simultaneous inhibition and excitation were given for longer periods, the two types of rebound described above must be distinguished.

In one case, that when simple inhibition was given, the resulting facilitation of the action currents was similar to that upon excitation alone. Maximum facilitation of action currents was obtained after 1½ min. (in many cases). This type of experiment is, quantitatively, quite sensitive to the conditions under which it is performed. For instance, if the preparation had been even slightly fatigued, the maximum facilitation of the action currents was smaller and tended to occur earlier. We also noticed marked seasonal variations. However, the results were always qualitatively the same. If simple inhibition was continued for longer periods, the action currents decreased again, becoming fatigued. Usually the height of the action currents and that of the contraction after release of inhibition bore a rough proportional relationship, but that was not always so. In some preparations the action currents were greatly fatigued when the contraction upon release of inhibition was still quite facilitated (Fig. 11). The same phenomenon could be seen in preparations where the exciter but not the inhibitor was stimulated for long periods.

Where supplemented inhibition was given, the rebound of action currents and contraction was always greater than in the previous example for a given period of excitation and inhibition when that period was longer than about 30 sec. The maximum rebound occurred after 2–3 min. of supplemented inhibition. When longer excitation and inhibition were given, fatigue occurred as in the previous case.

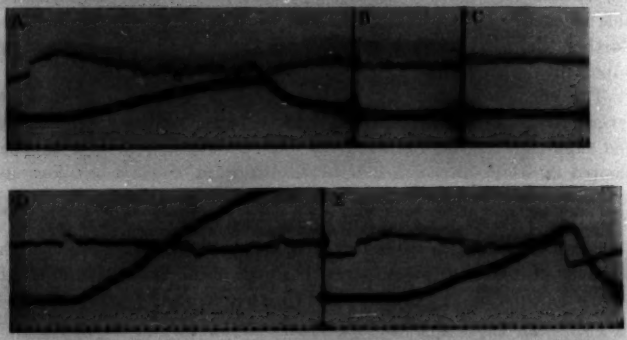


Fig. 11. Action currents and contractions (auxotonic) from a preparation to which prolonged simple inhibition is applied. A shows the initial contraction before inhibition is started. B and C give the action currents after 1 and 2 min. of simple inhibition respectively. In D the inhibition is turned off after 2½ min. There it will be seen that while the electrical response is very small, the contraction is facilitated. E is taken after a 1 min. rest; while the action currents have only partially recovered, the contraction is still facilitated. Time $\frac{1}{5}$ sec.

If, in either case, a short rest (2 min.) period were given when the contraction upon release of inhibition was quite depressed, the contraction was usually markedly facilitated upon resumption of excitation. This demonstrates that facilitation goes on notwithstanding fatigue or inhibition. Therefore fatigue is not the "opposite" of facilitation.

Discussion

From the results presented in this and other papers referred to, it is possible to distinguish at least five processes concerned in the excitation of the slow contraction of crustacean muscle. These are (1) the nerve impulse; (2) the facilitation and transmission between the nerve impulse and (3) the muscle action current; and (4) another facilitation and transmission linking (3) with (5) the contractile process itself. Our

experiments show that the impulses carried by the inhibitor fibre can set up processes which are able to block this chain in two distinct places. It will be indicated below that these two places may very well be the two transmission steps.

It seems quite certain that the motor impulse is transmitted to the muscle action current process by a mechanism which can be facilitated. This facilitation must be previous to the electrical response of the muscle since, as has been repeatedly pointed out, it goes on whether the muscle action current is reduced or not. Concerning the intimate nature of this facilitation-transmission mechanism there is little information. It is to be inferred, from the existence of facilitation and the absence of after-discharge, that facilitation does not imply transmission, so that these two processes are to be distinguished from one another [see also Katz, 1936].

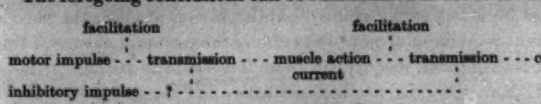
Wiersma & van Harreveld [1938] have pointed out that the muscle action current is most likely neither propagated nor all-or-none in the usual sense. However, it is indicated that the action current is an integral part of the excitation system rather than a mere side reaction. The results of prolonged supplemented inhibition as compared to those of similar simple inhibition offer a strong argument for this view. Here there is a distinct correlation between maximal facilitation of the electrical response and that of the contraction, although these two are certainly not directly proportional.

The fact that the action currents, after a period of prolonged supplemented inhibition, can rebound to a size they could not have attained otherwise, must result from a prevention of fatigue during their reduction. It seems certain that the electrical response is the first process in the excitation chain to be fatigued [Wiersma & van Harreveld, 1938]. Since the height of the action currents must be largely determined by the interaction between their facilitation and their fatigue, it is clear that the more efficient the reduction caused by supplemented inhibition is, the more effective the facilitation process will be.

A second facilitation process between the muscle action currents and the contractile process has been postulated [Wiersma & van Harreveld, 1938]. The best evidence from the data of this paper for a second facilitation is drawn from experiments with prolonged simple inhibition: after release of inhibition the action currents may be barely appreciable but the resulting contraction disproportionately high. It seems possible that the transmission here is accomplished by means of a chemical substance against which a possible inhibiting substance acts.

When the frequencies of excitation and inhibition are so adjusted that "just complete" inhibition occurs one could suppose that the inhibiting substance "neutralizes" the transmitter. A similar mechanism might be assumed for the other transmission process, but the difficulties in giving a satisfactory picture for that case are much greater since the reduction of the action currents is so extremely sensitive to time relations.

The foregoing conclusions can be summarized in the following scheme:


facilitation
facilitation
motor impulse - - - transmission - - - muscle action - - - transmission - - - contraction
current
inhibitory impulse - - ? -

We are unable to say anything definite at this time about the intermediate processes which link the impulse in the inhibitory axone to the two steps in the excitation chain which it blocks. However, we have pointed out that there is a definite but briefly accomplished facilitation in the reduction of the action currents at the beginning of supplemented inhibition. We would also remind the reader of the definite facilitation in inhibition of the mechanogram found in the abductor of the claw of *Eupagurus* by Wiersma & van Harreveld [1934].

Concerning the nature of the after-effect which appears when inhibition is given alone, we have no information to offer. It certainly reduces both the electrical response and the contraction, but whether or not its primary effect is on the former process, which then limits the extent of contraction, is uncertain.

Many of the obscure steps may become better understood after the study of the inhibition in other muscles and in other crustaceans. We have pointed out that in the crayfish the ratio R_e varies from muscle to muscle and is also different for the fast and the slow systems of a given muscle; the reason for these differences presumably depends on quantitative differences in the processes involved. Thus it is likely that effects which are here barely demonstrable or even absent (facilitation of inhibition of the contraction) will be distinct elsewhere.

Summary

The mechanism of peripheral inhibition in muscles of the crayfish was investigated by stimulating the exciter and the inhibitor axones separately. The ratio of frequency of inhibition to that of excitation necessary for "just complete" inhibition was determined for various muscles of the cheliped. This ratio differed widely from muscle to muscle

and was also different for the "fast" and the "slow" contraction of one muscle. For the abductor muscle it was found that the ratio was fairly constant for different frequencies of excitation.

The action currents during inhibition of the abductor were investigated. These action currents are reduced when the inhibiting impulse reaches the muscle shortly before the exciting one (supplemented inhibition) but not when the times of arrival are further separated. The reduced action currents may be as low as 20 p.c. of the unreduced height. No electrical sign from the muscle could be found when the inhibitor alone was stimulated.

However, when the inhibiting axone is faradically stimulated for a certain time it exerts a depressing effect on both the action currents and the contraction produced by an excitation given shortly afterwards.

It was found that continuous faradic stimulation of the inhibitor fibre could produce complete mechanical inhibition for more than an hour.

During inhibition it was demonstrated that facilitation of the action currents and the contraction continues at the same rate as when excitation alone is given. Facilitation is manifested to a marked extent in rebound after a prolonged period of supplemented inhibition. This is presumably due to prevention of fatigue of the action currents by their reduction.

It is concluded that inhibition can block the neuro-muscular transmission in two places. A scheme for peripheral excitation and inhibition of crustacean muscle is offered.

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J. Physiol. (1938) 93, 194–205 612.741.15

CHANGES IN MUSCLE CONTRACTION CURVES PRODUCED BY DRUGS OF THE ESERINE AND CURARINE GROUPS

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(*Received* 28 *February* 1938)

Eserine in small doses acts as a stimulant to muscle. This paper describes the depressant effect of large doses on the contraction curves of normal mammalian skeletal muscle and compares the eserine type of depression with that seen in curarine poisoning. Reversal of normal grading and alteration in the initial curve of contraction are the main characteristics of changes caused by drugs of the eserine group. These effects, as seen in a mixed muscle, quadriceps of the cat, were briefly described in a preliminary communication [Briscoe, 1936*a*]. The well-known antagonism [Pal, 1900] between eserine and curarine was also demonstrated by the rapid removal of either type of depression by its antagonist, thus restoring the muscle curves to the normal shape [Briscoe 1936*c*]. In these experiments muscle contractions were recorded by an isotonic method. In the present investigation isometric recording has also been used, and in addition the effect of these drugs on a red muscle, soleus of the cat, and a white muscle, gastrocnemius, has been tested. In all essential features the results have been similar whether recorded by isotonic or by isometric methods.

Methods

For isometric recording a flat steel spring $\frac{3}{8}$ in. wide firmly held in a robust steel frame has been used. The leg is first denervated by section of nerve trunks. The lower part of the muscle with its tendon and the piece of bone to which it is inserted is freed. Stout drills transfix the ischial tuberosity, the lower end of the femur and, when necessary, the tibia. These drills are clamped to steel pillars fixed to the operation table, the muscle being arranged to give a horizontal pull. The tendon is connected

by a steel hook to a short vertical rod attached to the flat spring. The changes in tension are recorded by a light aluminium lever moving in the vertical plane.

For isotonic recording the muscle tendon has not been freed, but the movement of the limb, whether extension of the knee or extension of the ankle, has been recorded by a thread passing from the limb to a light isotonic lever.

Both methods have their advantages and disadvantages. The isometric record gives a better picture of the changes occurring in the muscle during the initial stages of contraction, the isotonic curve being complicated by the overshoot of the limb which accompanies the sudden shortening of the muscle. On the other hand, the ease and quickness of the isotonic set-up allows simultaneous investigation of several muscles. In some experiments the effect of a drug on all three types of muscle has been shown in the same preparation. With suitable stimulation the isotonic preparation is practically unfatigable. The quadriceps can hold the leg in full extension for hours if allowed to shorten under its normal conditions of attachment [Briscoe, 1931], while under isometric conditions the contraction curve begins to give way in a few seconds (Fig. 2A). One reason for this difference is that the fusion rate under isometric conditions is higher than for isotonic recording. Owing to this greater liability to fatigue, the isometric preparation cannot be tested so frequently for its responses to different rates of stimulation. With drugs which act slowly this is no particular disadvantage, but with drugs like the onium salts which act in a few seconds and recover in a minute or two the isotonic method is useful.

For stimulation neon lamp discharges [Briscoe & Leyshon, 1929] have been used, a number of resistances with cut-out keys being inserted into the charging circuit to enable the stimulation rate to be varied in steps as desired without alteration in the strength of the stimuli. The nerves are placed on protected silver wire electrodes. Dial has been used as anæsthetic. Occasionally decerebrate preparations with preliminary ether and no dial have been used and similar results obtained. The circulation is kept intact. The drugs are injected into the jugular vein.

Results

Normal curves. In unpoisoned muscle a rise of stimulation rate (up to a certain point) produces an increased shortening or a greater rise of tension according to the method of recording employed. This increased

response to a higher frequency is referred to as normal grading. Still higher frequencies produce a deterioration in the responses and these have not been dealt with.

The first step is to find the lowest frequency compatible with fusion. (The variables upon which fusion rate depends have been discussed in a previous article [Briscoe, 1934].) The effect of increasing the stimulation rate is tested until improvement in the response ceases. This frequency is termed the optimum stimulation rate. It is usually in the neighbourhood of 100 per sec. for quadriceps and gastrocnemius and well below 100 per

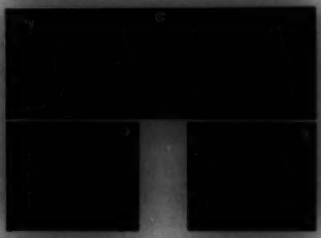

Fig. 1. Cat. Isometric curves of normal muscle. *G.* Gastrocnemius. Stimuli at fusion rate on the left, at optimum rate (90 per sec.) on the right. *Q.* Quadriceps. Rate (40 per sec.) just below fusion. *S.* Soleus. Rate (22 per sec.) just above fusion. Time interval, seconds. Tension calibration in kg.

sec. for soleus. In the controls responses to four or five different rates are recorded, but when quick changes are taking place, as in the restoration produced by the antagonism of curarine to eserine, it is advisable to test only one slow rate and one fast rate. Normal isometric curves are shown in Fig. 1.

Effect of drugs of the eserine group

Changes in the myogram produced by this group were first noticed after a depressant dose of prostigmin. It was established that eserine itself gave the same type of reaction and after this most of the experiments were carried out with prostigmin and a closely allied compound, substance 36 (methyl-phenyl-carbamic ester of 3-oxyphenyl-trimethyl-ammonium-methyl sulphate). Substance 36 can be tested without giving atropine. Atropinization (1–2 mg.) does not prevent the results described below.

The first stage of eserine poisoning is seen in the fast rate myograms. The response becomes smaller and the power of maintaining a contraction is diminished. As the poisoning deepens this deterioration in size and power

of maintenance becomes more marked, until eventually the magnitude of the response is equal to or smaller than the magnitude of the slow rate response. Normal grading is thus disturbed or reversed, the optimum rate of stimulation shifting from 100 per sec. (*circa*) to 50–75 per sec. in quadriceps and gastrocnemius; from 50–75 per sec. to 20–30 per sec. in soleus [Briscoe, 1936*b*, 1937]. Fast rates are occasionally tested before slow rates to show that the disturbance in grading is not the result of fatigue (Fig. 3B).

After the alteration in the fast rate myograms has been in evidence for a few minutes another change begins to show itself in the responses to the slow rate. The initial contraction which may or may not be diminished in size is followed by a brief period of depression which in turn is succeeded by a rise of tension or increase of shortening. This depression, followed by recovery, produces a notch in the myogram. In some preparations the notch appears early, soon after the first reduction in the size of the fast rate responses, in others it does not show until the fast rate (optimum for normal muscle) is producing smaller contractions than slow rates. If the stimulation is continued for more than a few seconds a gradual deterioration of the response sets in.

The course of events is illustrated in Fig. 2. In this experiment the drug acted quickly so that the first change is not shown. During the second minute after injection the fast rate response became smaller than the slow rate response. In the sixth minute there was the first indication of a notch and in the seventh minute this was obvious (Fig. 2C). This notch remained in evidence for nearly two hours except when it was temporarily removed in the manner described below. Two hours after injection the slow rate response was nearly normal but the fast rate response was still small and unable to maintain tension.

Varieties of deformed curves in different muscles are shown in Fig. 3. The abnormalities in these curves disappear gradually as the depressant drug is eliminated. The rate of disappearance varies greatly among different members of the eserine group. With substance 36 it is a matter of several hours and the course of elimination can be watched by recording the gradual restoration of the myograms. The curves can however be rectified rapidly by two methods: (*a*) very temporarily by repeating short closely spaced spells of stimulation, (*b*) more permanently by giving an antagonistic dose of curarine.

(*a*) In studying temporary rectification of deformed curves it is necessary to use a slowly eliminated drug like substance 36, so as to obtain a period when a given stimulation produces a constant effect.

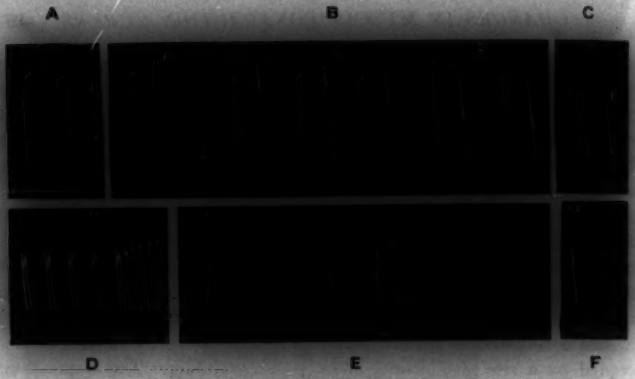

Fig. 2. Quadriceps. Isometric records. Figures above give interval after injection. Time, seconds. A. Effect of prolonged stimulation (20 sec.) at fusion rate, normal muscle. B. Slow and fast (50 and 125 per sec.) rates alternated in two-second spells. At arrow 0·5 mg./kg. substance 36 injected intravenously. In the second minute grading is reversed. C. In seventh minute notch in slow rate response. Lower line all at slow rate of stimulation. D. Effect of decreasing interval between spells of stimulation. E. After 8 min. inactivity; notched curve on fast drum. The second curve on the fast drum taken immediately after the four contractions shown on slow drum. F. One min. later, curve notched. Effect of prolonged stimulation.

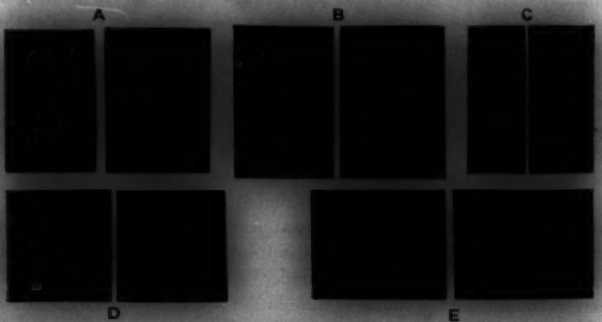

Fig. 3. Eserine and curarine types of depression. Time, secs. Upper line, quadriceps. All curves isometric. A. Rates 45, 75, 112, 160 per sec. Control curves first, then effect of substance 36. 1·0 mg./kg. Notch appears before reversal of grading is complete. B. Fast rates tested first (125, 80, 50 per sec.). Controls and then effect of substance 36. 0·5 mg./kg. C. Rates 40 and 100 per sec. Effect of curarine. 0·2 mg. Both responses proportionally depressed. Lower line. D. Gastrocnemius. Rates 35, 58, 90, 122 per sec. E. Soleus. Rates 22, 36, 55, 77, 110 per sec. Controls and then effect of substance 36. 1·5 mg./kg. D and E recorded in same experiment.

Short spells of stimulation lasting 1–2 sec. applied at intervals of 10–20 sec. are usually suitable for this purpose. When a series of uniform curves has been obtained the interval between the spells of stimulation is suddenly reduced to, say, 1 sec. The next response is different in that the notch is less well marked and the second or third response may show a normal curve with no initial depression and therefore no notch, though a slow depressant effect will appear if the stimulation is prolonged beyond a few seconds. After a short rest, abnormalities again appear, the notch becoming deeper the longer the period of inactivity (Figs. 2 and 5). The height of the initial contraction, if it has been reduced by the drug, is often improved by repeated activity and is reduced again by prolonged rest (Fig. 2E).

Fig. 4. Soleus. Stimulation rate 22 per sec. Time in sec. Curves deformed after substance 36. 1·5 mg./kg. Control curve shown on fast drum in Fig. 1. Upper line. Notch removed or restored by shortening or lengthening interval between spells of stimulation. Lower line. Notch permanently removed by curarine 0·15 mg. injected at arrow.

(*b*) A small dose of curarine is sufficient to abolish the notched curves seen in eserine poisoning. The rectification usually occurs in less than a minute and is long lasting. It is not easy to choose the dose of curarine which will correct the eserine effects with speed and exactitude and yet not give rise to its own characteristic depression. Fig. 4 shows the effect of 0·15 mg. of curarine chloride on the notched curve of soleus under isometric conditions. In Fig. 5 the abnormal quadriceps curve, recorded isotonically, is rectified in less than a minute by 0·6 mg. of curarine. The curves of soleus and gastrocnemius showed similar swift rectification in the same preparation. In both these figures (4 and 5) responses to a slow rate of stimulation are shown. With fast rate responses the antagonistic effect may be equally rapid; a figure showing the change, in less than a minute, from a small twitch-like response to a full size normal contraction curve has already been published [Briscoe, 1936*c*].

The chief difference between isometric and isotonic records is seen in the rapidity of the phases depression-recovery. If the muscle is contracting under isometric conditions this period may last from two to three seconds. Fig. 2E. If contracting isotonically the whole of the notch, depression and recovery, usually occurs in a second or less, though occasionally recovery is slow (Fig. 5). This applies to quadriceps and gastrocnemius. The choice of experimental method does not make much difference to the myograms of soleus, depression and recovery occur

Fig. 5. Quadriceps. Effect of substance 36, doses of 0·5 mg./kg. (i.v.) given at 12·47, 1·45 and 2·52 p.m. A. 3·8 p.m. Response to rate of 35 per sec., strength maximal for full extension. B. 3·9 p.m. Notch removed by repeated spells of activity which are shown on slow drum. C. 3.10 p.m. After one minute's rest notch reappears. D. 3.11 p.m. Notch shown on fast drum. 3.25 p.m. Curarine 0·2 mg./kg. E. 3.26 p.m. One minute after, notch nearly gone. Isotonic records, contraction downwards.

slowly whichever method is used. In both the notch is a temporary interruption in the steady increase of contraction, rather than a diminution in the size of contraction (Fig. 4).

Other members of the eserine group. A number of members of this group have been tested and have caused changes in the contraction curves similar to those described above. They vary considerably in strength, in rates of action and elimination. Substance 36 takes longer to exert its action than prostigmin, but on the other hand it is eliminated more slowly and thus possesses some clinical advantage in the treatment of myasthenia gravis. Substance 38 (dimethyl carbamic ester of 8-hydroxy-methyl-quinolinium-methyl sulphate) is more active than prostigmin and more quickly eliminated. Preparation 3393 (dimethyl carbamic ester of *m* hydroxy-phenyl-diethyl methyl ammonium iodide) acts with great rapidity in very small doses. A dose of 0·01 mg./kg. (after 1 mg. atropin) produces about the same effect as 0·25 mg./kg. of eserine; it is rapidly eliminated. (I am indebted to the makers, Roche

Products, for samples of these three drugs.) Miotin hydrochloride, kindly sent me by Dr Stedman, produces marked depression with doses of 0·25 mg./kg. showing reversal of grading and notched curves.

Hordenine compounds

Schweitzer *et al.* [1938] have shown that compounds of hordenine vary in their effect on the central nervous system. Prof. Wright has kindly sent me samples of two compounds, hordenine hydrochloride

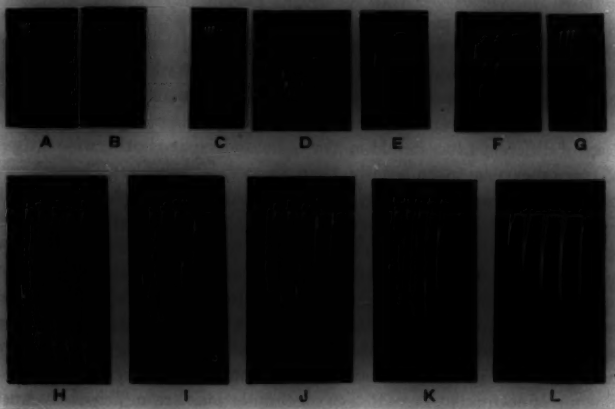

Fig. 6. Upper line, kneejerk. A. Control. B. Two min. after hordenine methyl sulphate 5 mg./kg. C. Control. D and E. Effect of hordenine hydrochlorine 8 mg./kg. F and G. Two min. and 27 min. after curarine. Lower line, quadriceps, isotonic record, contraction downwards. Both drugs give similar depressant effects peripherally. H. Control for rates (35, 58, 87, 122, 175 per sec.). I and J. Progressive depression after hordenine hydrochloride 8 mg./kg. Optimum rate shifts from 122 to 58 per sec. K. Two min. after curarine 0·45 mg. normal grading resumed. L. Twenty-five min. later. Typical curarine depression.

which is convulsant and hordenine methyl sulphate which is depressant for the C.N.S. The two drugs have identical anticholinesterase action *in vitro*, and the same peripheral effect, namely potentiation of single shock twitches, tested every 10 sec. I have tested the two drugs by the methods given above. Both produce peripheral depression of the eserine type, notching and reversal of grading being well shown (Fig. 6). On the other hand, they produce different effects on the kneejerk (tested in the opposite intact limb). Hordenine methyl sulphate (quaternary salt)

caused depression (Fig. 6*b*) or temporary abolition of the kneejerk, while hordenine hydrochloride (tertiary salt) caused excitement on the reflex side which was coincident with depression on the peripheral side. In one case the quadriceps became so rigid that it was difficult to obtain the "clasp-knife" reaction, there was tail movement, licking and swallowing; in another the kneejerk was nearly doubled and was followed by tonus (Fig. 6E). Curarine causes an immediate improvement in the peripheral depression.

Depressant effects of curarine on normal muscle

In mild curarine poisoning there is no reversal of grading, faster rates still produce larger contractions than slow ones, but magnitude and power of maintaining a contraction are reduced. With deeper poisoning the responses to all rates are twitchlike contractions much reduced in size but retaining their normal order relative to each other (Figs. 3C and 6L). Tests of different rates should not be too closely spaced otherwise fatigue effects supervene more readily than in normal muscle. They should therefore be taken occasionally in reverse and random order.

Discussion

The theory of chemical transmission of excitation assumes that the quanta of acetylcholine which are liberated by the arrival of impulses at the nerve endings act as direct stimulants to muscle fibres and are then rapidly destroyed by the cholinesterase at the nerve endings. It is known that eserine delays the normal swift destruction of acetylcholine, thus allowing of accumulations. If these accumulations are sufficient to cause reduction in size of contraction, it is to be expected that the responses to fast rates would be more deeply affected and at an earlier stage than the responses to slow rates of stimulation. Accumulations would reach the threshold for depression the more rapidly the faster the rate applied and this would account for the reversal of grading seen in deep poisoning.

Abnormal myograms of the same general type (contraction-relaxation-contraction) as those described here occur after eserinization in frog muscle [Cowan, 1936] and in the nictitating membrane [Brown & Feldberg, 1936; Cannon & Rosenblueth, 1937]. Brown & Feldberg measured the output of acetylcholine from the superior cervical ganglion perfused with eserine. During prolonged preganglionic stimulation the output starts at a high level and then falls rapidly to a steady low level. The slow recovery of contraction of the nictitating membrane after the initial depression occurs during the rapid fall in the output of acetylcholine. In skeletal muscle depression and recovery occur with

much greater rapidity, but the sequence of events is the same. Reduction in the size of initial contraction followed by a swift relaxation may be attributed to excess of acetylcholine. With fast rates of stimulation this relaxation persists. The recovery from the initial depression seen with slow rates may be explained on the ground that after the first excess has been worked off a more normal balance obtains between the formation and destruction of acetylcholine. The muscle begins to emerge from its depression causing a notch to appear in the myogram. This more normal balance may be due partly to a lowered output of transmitter, partly to the long interval between the stimuli, allowing more time for its destruction.

The disappearance of the notch after a short spell of activity is difficult to explain. If there is a drop in output with continued stimulation this condition may persist for a short period after stimulation ceases, so that a contraction occurring within this period would be normal because there was no excess of acetylcholine. The period of exemption is short, as depressant effects quickly reassert themselves. Brown & Feldberg discuss the possibility that acetylcholine is liberated from a pre-existing complex in which it is stored in an inactive form and protected from destruction. If this suggestion prove to be correct the exemption from depressant effects would be due to depletion of the depot and the rate of refilling could be gauged by their reappearance.

The differences seen in the myograms of eserine and curarine poisoning suggest that these two drugs produce their depressant effects by different mechanisms. The suggestion has been discussed in a previous publication [1936*c*] that curarine antagonizes eserine by raising its threshold for depressant action. If the dose of curarine is larger than is necessary to secure the antagonistic effect, the rectification of curves may occur within a few seconds, but is transient. After two or three minutes the curves again become abnormal, showing the curarine type of poisoning. With all rates there is loss of power to maintain a contraction and proportional diminution in magnitude, so that normal grading is resumed. It is difficult to form any conception of the mechanism by which the temporary release from depression is obtained. If a struggle took place for the same receptors, or if the drugs combined with receptors of different pattern, as suggested in the specific receptors theory of drug antagonism [Clark, 1937], why should there be a period of normal response? A rise of threshold for depressant effects would explain how the muscle can pass through a short period during which the curves are more or less normal before a rise in stimulation threshold brings about depression of a

different type. Something more than a simple rise of threshold is required to explain the selective action of the two groups of drugs. It is possible that they act at different sites in the nerve muscle junctions. Since both types of drugs, in large doses, depress the two functional activities of muscle, contraction and the maintenance of contraction, the selective action is largely a question of emphasis, and differences such as the thickness or the permeability of membranes may be of decisive importance.

It has been shown by Brown *et al.* [1936] and confirmed by Schweitzer & Wright [1937] that eserine potentiates the nerve muscle twitch when single shocks are applied at intervals of ten seconds. Records of muscle action potentials show that this effect is due to repetitive response of individual fibres to single nerve volleys [Brown, 1937]. With iterative stimulation as in the present experiments depression ensues after eserinization. It is clear that the type of response in peripheral muscle, whether potentiation or depression, can be varied by regulating the interval between the stimuli applied to the nerve. It follows that peripheral effects bear no necessary relation to the states of excitement or depression produced in the central nervous system by the various drugs.

Summary

Drugs of the eserine group produce abnormalities in the contraction curves of different types of mammalian muscle (mixed red and white). Normal grading is disturbed or reversed in that slow rates of stimulation produce larger contractions than fast rates.

Curarine produces abnormal myograms of a different type.

The abnormal contraction curves seen in eserine depression in response to slow rates of stimulation can be temporarily rectified by repeated activity of the muscle.

The antagonistic action of curarine to eserine is shown by the rectification of the responses to all rates. With correct dosage this rectification is permanent; if the dose is too large the period of normal response is followed by depression of the curarine type.

The possible mode of action of this antagonism is discussed.

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J. Physiol. (1938) 93, 206–214 611.83:611.314:612.392.013 A

DEGENERATIVE CHANGES IN THE AXIS CYLINDERS OF THE DENTAL NERVES, DUE TO DIETS DEFICIENT IN VITAMIN A AND CAROTENE

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(*Received* 12 *April* 1938)

During recent years the investigations of E. Mellanby [1926–35] and his co-workers have firmly established the truth of the observation that marked degenerative changes occur in the nerve fibres of both the central and peripheral nervous systems of animals which have been kept on a diet deficient in vitamin A. The histological methods which have been used to demonstrate these changes have been concerned mainly with the alterations found in the myelin sheath of the nerve, and the Marchi technique has been used for the most part for this purpose. On the other hand, little is known of the influence of such a diet on the structure of the axis cylinders themselves. Our lack of knowledge on this subject is due principally to the capriciousness of the methods which have to be employed in staining these structures. The silver impregnation techniques of Bielschowsky and Cajal are very uncertain in their action, and it is difficult with them to obtain consistent results.

A study of the literature reveals that very few of the workers in this field have employed an axis cylinder stain. Zimmerman & Burack [1934] used Bielschowsky to stain the axis cylinders of dogs which had been fed on diets deficient in vitamin B_1. Apparently they were unable to detect any changes in these structures even in fibres which showed myelin degeneration. Grinker & Kandel [1933] utilized both Cajal and Bielschowsky, and found no changes in the axis cylinders. It is also to be noted that these investigators were unable to detect any changes in the central nervous system in animals fed on diets deficient in vitamin A or vitamin B complex. Culley [1927], in an investigation into the polyneuritis of fowls, says that axis cylinders undergo changes at a very late

period, long after the myelin degeneration, but he gives no illustration of such changes nor does any description of them appear in his paper. Woollard [1927] utilized Bielschowsky to demonstrate nerve changes in starvation and beri-beri, and found degenerative alterations of the axis cylinders in both these conditions. He noticed particularly that marked changes occurred in the muscular nerve endings. These structures lost much of their complicated form, and indeed some of the nerve endings seemed to have disappeared altogether. More commonly, however, only the finer branchings were lost. The myelin sheaths of the fibres connecting the endings were often swollen and fragmented and the axis cylinders themselves sometimes became fragmented and even disappeared but, as a rule, they persisted even when the myelin was greatly altered. This worker also used the intravital methylene-blue stain, and obtained similar results with this method. The changes in the nerve endings affected the muscle spindles as well as the motor nerve endings. Woollard also found that the degenerative processes were more marked peripherally and became less distinct when the nerve fibres were traced centrally towards the spinal cord. In the larger trunks it was difficult to detect any affected fibres. Mellanby [1935] experimented with several silver methods, but he states that the results which he obtained, especially in the early stages of axis cylinder degeneration, were not sufficiently definite to warrant publication. However, in rabbits fed on vitamin A and carotene-deficient diets, changes in the axis cylinders were definitely present before advanced myelin degeneration occurred.

From this brief survey of the literature, it will be seen that little is known of the degenerative processes which occur in the axis cylinder of nerves of animals on a vitamin-deficient diet.

Recently, Lewinsky & Stewart [1936*a*, *b*, 1937*a*, *b*] have made extensive investigations into the innervation of the dentinal structures and the neighbouring tissues and, in these investigations, they relied practically entirely upon the Cajal silver impregnation method to demonstrate the course and distributions of the nerve fibres. They found that the decalcification that is necessary before it is possible to prepare histological specimens from this material had profound effects upon the staining properties of this method. These effects were investigated by Gooding & Stewart [1934] who discovered that nitric acid, which is normally employed as a decalcifying agent, delayed the action of the silver nitrate, and it took longer to impregnate the nerve fibres. On the other hand, it had one very useful characteristic. It checked to a very marked degree the formation of deposit in the non-nervous tissue, which so commonly

occurs in specimens which do not require decalcification. This deposit is sometimes very marked, and may conceal the finer fibres and make it difficult or even impossible to trace their course and distribution. When, however, nitric acid was employed for decalcification, these other tissues were stained uniformly yellow or orange in colour, and gave a marked contrast to the black- or darkly brown-stained axis cylinder. Also, the coarse deposit was either absent or much reduced in amount. They also found that 48 hr. immersion of the tissues in 5 p.c. nitric acid seemed to be the optimum time to prevent deposit, without unduly checking the impregnation of the nerve fibres. These authors therefore recommended as a modification of the Cajal technique, that after the tissues had been fixed, they should be immersed in nitric acid for 48 hr. before being placed in the silver nitrate solution. The results obtained by this method were so satisfactory and consistent that it was felt that this was a suitable medium to employ as a routine in the investigation of the axis cylinder changes which occur in vitamin A deficiency. Rats were selected as the experimental animals and, from the histological viewpoint, they had this great advantage, that the tissues of rodents stain particularly well with this modification, and secondly, the lower jaws of these animals took about 48 hr. to decalcify.

Methods

Experimental feeding of the rats was begun at weaning, when the animals were 3 weeks old and weighed about 30 g., and continued for varying periods up to 384 days.

Basal diet. Oatmeal, 89 g., bakers' yeast, 5 g., heated alfalfa,[1] 2 g., calcium carbonate, 1·5 g., sodium pyrophosphate, 1·65 g., sodium chloride, 0·85 g. Each rat received 10–15 g. of this mixture daily, to which was added 0·5 c.c. of heated olive oil,[1] 0·3 c.c. of orange juice and 125 international units of irradiated ergosterol (vitamin D). Instead of the above, a few animals were given the casein-starch basal diet previously used by M. Mellanby & King [1934]. Vitamin A in the form of mammalian liver oil was added to the rations of some of the rats as shown in Table I.

After the animals had been killed with chloroform, half of the lower jaw was retained at Sheffield and fixed in 10 p.c. formol saline to determine the general tissue changes, and the other half was placed in a tube containing ammoniated alcohol and forwarded to Manchester. After 48 hr. in this solution, the half jaws were placed in nitric acid for a further 48 hr. and were then found to be completely decalcified.

[1] Heated and oxygenated so that any traces of vitamin A were eliminated.

The specimens were next passed through the various stages of the Cajal silver impregnation method and finally dehydrated and embedded in paraffin. Longitudinal serial sections at a thickness of 12μ were cut parallel to the vertical plane of the half jaw, with the teeth *in situ*. The cutting of these serial sections offers little difficulty, and they are particularly valuable because the course of the fibres and small bundles can be followed from one section to another for long distances. Having the half jaw, it was also possible to study differences in the condition of the axis cylinders in different regions and tissues of that part.

Results

The general clinical findings just before death and the vitamin A content of the liver are shown together with the dietary variations and experimental period in Table I. Confirmation has been obtained of the previous observation of M. Mellanby & King [1934] with regard to the breaking down of the myelin sheaths of the afferent dental nerves of rats fed on diets deficient in vitamin A and carotene, when stained by the modified Marchi technique of R. J. Stewart [1936].

On histological examination of the nerves, it was found that the condition of the axis cylinders was not uniform throughout the tissues. In the pulps of the molar teeth, degenerative changes of the nerves were particularly marked, and in different specimens many stages could be observed from quite early ones up to those of an advanced degree. In those pulps in which the degenerative conditions of the axones were most pronounced, the connective tissue was also undergoing change. Signs of degeneration were also detected in the nerves of the periodontal membrane, but these changes were not so marked as those found in the pulps themselves. On the other hand, no changes of any kind could be detected in the nerve supply of the mucous membrane of the gum. In some of our specimens, small portions of the muscles still remained, and although not sufficient to give indications of alterations in the nerve endings themselves, there was sufficient tissue present to show that the fibres of the motor nerves themselves were little, if at all, affected.

As the process of degeneration was most clearly seen in the pulps of the molar teeth, our description of these changes is confined to the nerves of this tissue. The earliest signs which were observed during the course of this investigation consisted in a definite thickening of the axis cylinders. This is clearly demonstrated in Pl. I, Figs. 1 and 2, which are photomicrographs of nerve bundles running in the pulp tissue and are of the same magnification, namely 233, as are all the photomicrographs in

TABLE I

Rat no.	Basal diet	Diet addition	Duration of experiment (days)	Condition of animal immediately prior to death	Vitamin A contents of liver (blue units per g. liver)*
2582	Oatmeal, etc.	—	74	Stance and movements appear normal; eyelids slightly inflamed; lower incisors normally pigmented	Not estimated
2585	Ditto	Vitamin A, 1500 international units daily	74	No abnormalities seen	Not estimated
2581	Ditto	—	147	Slightly shaky; no eye symptoms; lower incisors normally pigmented	23
2583	Ditto	Vitamin A, 1500 international units daily	147	No abnormalities seen	2517
2579	Ditto	—	203	Hind legs rather stiff; head shaky; movements slightly uncertain; lower incisors rather pale	0
2578	Ditto	—	234	Very high on legs, stiff and shaky; body hunched; eyes partly closed with sore lids; very weak	0
2577	Ditto	—	238	High on legs, stiff and rather shaky; eyes partly closed, cornea of right eye dull; cornea of left eye slightly ulcerated; lower incisors pale	0
2580	Ditto	—	289	Very stiff, shaky and weak; both eyes almost sealed up; eyelids very sore; both corneas ulcerated; coat poor; lower incisors very pale	0
2584	Ditto	Vitamin A, 1500 international units daily	289	No abnormalities seen	941
2303	Casein, etc.	—	384	Shaky and unsteady; slight head movements; cornea of each eye opaque and swollen; lower incisors pale	0
2300	Ditto	Vitamin A, 1500 international units daily	384	No abnormalities seen	19

* As estimated by the antimony trichloride method, using a Rosenheim-Schuster (no. 63) colorimeter.

this paper. Pl. I, Fig. 1, is the dentinal pulp from one of the control rats (no. 2585). As in other parts of the body, the nerve fibres in the pulp fall into two groups, namely, thick and thin fibres and the bundle which is illustrated in this figure is formed of thick fibres of a normal structure. Pl. I, Fig. 2, is from rat 2581, which had been fed for 147 days on a diet deficient in vitamin A. This bundle also consists of the thicker type of fibre, and a glance is sufficient to show that its individual fibres have increased markedly in breadth and their diameters are far greater than those of any seen in a normal pulp. An examination of the cornual region of the pulp demonstrates the changes which occur in the finer

Fig. 1.

Fig. 2.

Fig. 3.

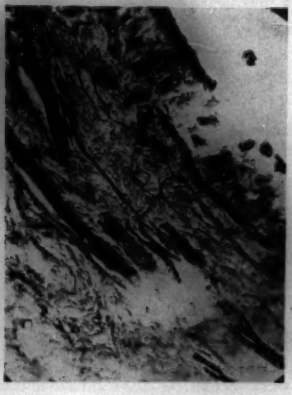

Fig. 4.

To face p. 210

Fig. 5.

Fig. 6.

Fig. 7.

Fig. 8.

fibres. Pl. I, Fig. 3, is a photomicrograph which was taken from this region in the same control rat (no. 2585). In this portion of the pulp the number of thick fibres is markedly reduced and the finer fibres greatly increased in number, largely due to the thick fibres dividing into arborizations. Pl. I, Fig. 4, is also from the cornual region, but this rat (no. 2578) had been on diet for 234 days. A definite alteration will be seen of a similar nature to that already described in the main bundles lower down in the pulp. The main fibres are distinctly thickened, and there is a definite increase in breadth of many of the finer ones. Besides this general condition, there are numerous irregular swellings in the course of the finer fibres which bear a close resemblance to the end bulbs which were seen in normal nerves, but they are much larger. Some of these on examination were found to contain a lighter area in the centre, giving indications of the beginning of vacuole formation. A good example of such a swelling with a clear centre will be seen in Pl. II, Fig. 4 itself.

The next stage, foreshadowed in the last, is the actual formation of vacuoles in the course of the thicker fibres. This can be seen in Pl. II, Fig. 5. This photomicrograph was obtained from the cornu of the pulp from rat 2579. This specimen is a further illustration of the marked thickness of the fibres which is clearly brought out when it is compared with the normal in Fig. 3. These vacuoles continue to increase in size, and in some examples appear to have lost the argentophil substance. In the next stage, many of the vacuoles break down altogether, leaving the fibres rough and serrated and, when several fibres are grouped together in a bundle, they have the appearance of a network, rather than a fascicule. An example of this is shown in Pl. II, Fig. 6, where the curious network-like appearance of a small bundle in the pulp can be seen. It should be compared with a normal bundle in Pl. I, Fig. 1. This degenerated bundle, after a short course, divides and the isolated individual fibres will in some parts be seen to contain large fibres, but in others the vacuoles have ruptured and given the fibres the serrated appearance mentioned above, which is very different from the smooth outline of the normal nerve.

This is as far as it has been possible to trace the effects of vitamin A deficiency on the nerves in the molar pulps, with the material at present at our disposal, but it is quite possible that the nerve fibres may ultimately disappear altogether and further experiments are now in progress which, it is hoped, will supply the evidence to determine this point.

It has already been mentioned that these changes were not uniform throughout the jaw, and that in our specimens they have been most strongly marked in the pulps of the teeth and have also been present to

a somewhat lesser extent in the periodontal membrane. The nerves supplying the gum, as distinct from the subgingival epithelium have, however, been unaffected (Pl. II, Fig. 7), even in those specimens where degenerative changes in the nerve fibres of the pulp are far advanced (Pl. II, Fig. 8, which is a photomicrograph of the pulp of a tooth from the same jaw as Pl. II, Fig. 7). It will be noticed (Table I) that rat 2300 showed no clinical signs of vitamin A deficiency but that at death the vitamin content of the liver was decidedly low. Moreover, examination of the pulp nerves of this animal showed early degenerative changes in the axis cylinders. Since the rat had received a liberal supply of vitamin A throughout the experimental period, it would seem that some unknown factor had rendered defective the absorption or utilization of the vitamin.

Discussion

The degeneration of the nerve fibres which have been separated from the nerve cell, has been fully described by Cajal [1928]. It is an acute process in which the degeneration occurs very rapidly and bears little resemblance to the slow chronic degeneration which has been described in this paper, and therefore requires no further discussion at this point.

Of much greater significance is the work of Speidel [1935]. This author has written a valuable contribution to our knowledge of the changes which take place in the nerve fibres during the earliest stages of irritation and degeneration. The study was made on the nerves of the tails of living frog tadpoles, and the changes were observed by direct microscopic examination and cinephotomicrographs. He found that the irritation of a nerve fibre by an adjacent wound, among other interesting features, had a tendency to cause the axis cylinders to lose their smooth edge and become slightly wavy. This apparently is the earliest stage which takes place in the axon cylinder change, and has not been observed by us very clearly in the present investigation, but certain of our specimens do suggest that such a stage does occur before the swelling of the fibres. In the irritative changes in a myelin nerve after alcohol, Speidel found specimens in which fine fibres ended in spherical knobs containing small vacuoles. He gives an illustration of this in his paper and it resembles very strikingly the condition which we have described in Pl. I, Fig. 4. When Speidel's animals were embedded in ice for over an hour, marked tissue irregularities developed in the more delicate parts of the tail fin. There was strong nerve irritation, including swelling, vacuolization and tortuous course of the axis cylinder. We have not

observed any increased tortuosity in the course of the nerve fibres in our specimens, but the swelling and the vacuolization of the fibres closely correspond to our findings. It would appear, therefore, that the degenerative changes in the axis cylinder in vitamin A deficiency correspond to those which occur when the nerve fibres are acted upon by irritants either of a chemical or of a physical nature.

E. Mellanby [1935] found that when the amount of degeneration in the myelin was only "annular", the nerve fibres so affected could be brought back to normal fairly rapidly when adequate vitamin A was supplied in the diet. If, however, the degeneration was more advanced, and the myelin sheath had disintegrated, structural recovery required a much longer time. These changes described by Mellanby resemble the irritative changes in the myelin sheath seen by Speidel, and we have already shown that the changes in the axis cylinder after vitamin A deficiency also resemble those seen by Speidel. It is probable, therefore, that when there is only annular degeneration of the myelin, there will be but little alteration in the axis cylinder, probably confined to thickening. These conditions could be remedied by a diet adequate in vitamin A. It is probable that the later changes in the axis cylinder correspond with those cases in which the myelin is markedly degenerate, and do not respond so effectively to treatment by administration of vitamin A.

Further investigations are in progress into the possible relationship between structural changes occurring in the epithelium and nerves of the subgingival tissue due to vitamin A deficiency. Abnormalities in the calcified tissues of the incisor teeth of A-deficient rats were demonstrated by one of us (J.D.K.) at the Annual Meeting of the British Dental Association [1937]. They will be described in a subsequent report together with changes found in the subgingival epithelium adjacent to the molar teeth and in the alveolar bone. A brief account has already been given of the effects of vitamin A deficiency on the development of the teeth and jaws in dogs [King, 1936].

SUMMARY

1. The effects of deficiencies of vitamin A and carotene upon the axis cylinders of the dental nerves of rats have been investigated.

2. The nerves of the pulp were markedly affected and the nerves of the periodontal membrane showed somewhat less severe changes.

3. An account is given of the degenerative changes which were found in the nerves of the pulp.

The expenses of a portion of this investigation have been defrayed by the Medical Research Council, and one of us has also received a whole-time grant from the Council, for which our thanks are due.

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EXPLANATION OF PLATES I AND II

Plate I

Fig. 1. Rat 2585 × 233. Normal bundle of nerve fibres.

Fig. 2. Rat 2581 × 233. Nerve bundle showing early thickening.

Fig. 3. Rat 2585 × 233. Distribution of normal nerve fibres in the cornu.

Fig. 4. Rat 2578 × 233. Thickening of nerve fibres, swelling and early vacuole formation in the cornu of the pulp.

Plate II

Fig. 5. Rat 2579 × 233. Further thickening and more marked vacuole formation in the cornu.

Fig. 6. Rat 2577 × 233. Late degenerative changes in nerve bundle in the pulp.

Fig. 7. Rat 2302 × 233. The gum and normal nerve fibres.

Fig. 8. Rat 2302 × 233. Late degenerative changes in nerve bundle in the pulp.

J. Physiol. (1938) 93, 215–262 612.817.1

THE ACTION OF ESERINE-LIKE AND CURARE-LIKE SUBSTANCES ON THE RESPONSES OF FROG'S NERVE-MUSCLE PREPARATIONS TO REPETITIVE STIMULATION

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(*Received* 6 *May* 1938).

The experiments to be described in this paper were the first step in some work which was begun in April 1935, after Dale & Feldberg [1934] had suggested that acetylcholine might be the chemical transmitter at motor nerve endings, and which has been partly summarized in previous notes [Cowan, 1936 *a*, *b*, *c*]. The work is based on the following considerations.

Several studies of electrical responses showed that, normally, a single impulse in a motor nerve fibre sets up a single propagated (all-or-none) impulse in each of the muscle fibres which it supplies, no matter whether the nerve impulse is an isolated one or one out of a short series having a frequency of 100 per sec. Therefore, if ACh. is the transmitter its action must be limited to a region small in comparison with the size of a muscle fibre. By analogy with electrical excitation it seemed probable that, at the part of a muscle fibre juxtaposed to its motor nerve ending, the concentration of the ACh. liberated by each nerve impulse would have to attain a threshold value to initiate a propagated impulse from there; also, that in the intervals between the successive nerve impulses during a tetanus the concentration of ACh. would have to fall below that threshold. The last-mentioned requirement might be achieved through the hydrolysis of ACh., probably accelerated by choline-esterase, which,

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according to Stedman *et al.* [1932], is the enzyme specific for the reaction; by diffusion into the remainder of the muscle or interfibrillar spaces; by a combination of the foregoing agencies. Numerous experiments on the production of, and recovery from, "neuro-muscular fatigue" with isolated preparations, mainly frog's, testify that a third possible agency—diffusion into the blood stream—plays no essential role.

If the hypothesis that ACh. is the transmitter were accepted, then the reversal of curare paralysis by eserine [Rothberger, 1901; Langley & Kato, 1915], by miotine [Stedman & White, 1931], and by "Prostigmin" [Aeschlimann & Reinert, 1931], could be explained in two ways. (Hereafter this last compound will be called "prostigmine" to conform with the usual chemical nomenclature.) Brinkman & Ruiter [1924, 1925] perfused frog's hindlimbs with Ringer's solution, and found that when the (mixed) nerves supplying the muscles were stimulated an ACh.-like substance could be detected in the perfusate. They also found that the liberation of the ACh.-like substance still occurred when sufficient curare to prevent the motor impulses from causing contraction was present. The implication of this experiment was that curare does not influence the liberation of the transmitter. After studying the curare-like action of a number of quaternary ammonium cations Ing & Wright [1931; also Ing, 1936] suggested that paralysis of neuro-muscular transmission involves a permutite-like exchange at motor nerve endings. Since ACh. and curarine, the active alkaloid of curare (for references see Boehm, 1920), are both quaternary ammonium cations it seemed that paralysis by curarine might be due to blocking of the region on which ACh. normally acts. (If, as the recent work of King [1935] on tubocurarine suggests, curarine is not a mono- but a di-quaternary salt the argument would be unaffected.) One explanation of the reversal of paralysis by the three compounds mentioned was that they might inhibit choline-esterase at the region of the nerve ending and so enable the liberated ACh. to compete better with curarine for the part of the muscle juxtaposed to the nerve ending. Eserine had been shown to have the power of inhibiting choline-esterase [Engelhardt & Loewi, 1930; Matthes, 1930], and miotine had been shown to have the same power [Stedman & White, 1931; Stedman & Stedman, 1932]. The alternative explanation was that the three drugs lowered the threshold, either of the whole muscle, or only of the part juxtaposed to the nerve ending, to ACh.

Since it was not clear from previous investigations how far eserine influenced nerve and muscle as well as neuro-muscular transmission

[for references and discussion see Langley & Kato, 1915] the particular aims of the first step of this work were as follows: To examine, in conditions simplified to exclude as many complicating factors as were known: (1) the actions of eserine and of prostigmine upon the responses of nerve, of muscle, of nerve-muscle preparations, to repetitive stimulation at various frequencies; (2) the actions of a number of simple synthetic compounds having certain eserine-like properties, in the hope that it would be possible to demonstrate a parallel between action on neuro-muscular transmission and inhibitory action on choline-esterase; (3) the antagonisms, where existing, between eserine and eserine-like substances on the one hand, and curarine and certain quaternary ammonium salts on the other hand. (Hereafter the expression "eserine-like substances" includes eserine itself, and similarly any reference to curare-like substances includes curarine.)

Apart from any possible action of eserine-like substances on nerve, the differences between the myograms in response to (maximal) direct stimulation of muscle and to the stimulation of muscle through its nerve appeared to provide a simple method of estimating the effectiveness of neuro-muscular transmission. It was clear that if the errors of such a method were to be kept small a muscle in which all the fibres could be stimulated directly without danger of stimulating the intramuscular nerve twigs was desirable. Whilst it was clear also that a blood supply would have been desirable to minimize muscular fatigue, and might perhaps have been arranged so as to exclude the excretion and possible metabolism of the eserine-like and curare-like substances, the contingency that the blood cells or plasma proteins might adsorb relatively large amounts of those substances and so vitiate the attempt to make the experiments quantitative could not be excluded. I decided therefore to dispense with a blood supply and to use a muscle sufficiently thin for oxygen (or drugs) to diffuse in, and for metabolites to diffuse out [Hill, 1928; Hill & Kupalov, 1929]. The frog's isolated sartorius muscle immersed in Ringer's solution heavily buffered with bicarbonate and well stirred with oxygen-5 p.c. CO_2 mixture was selected as the nearest practical approach to the requirements of this work. The isolated nerve-sartorius preparation was inferred to have the advantage also that the nerve endings were readily accessible, since large quaternary ammonium ions, including curarine, had been shown to paralyse it rapidly [Ing & Wright, 1931, 1933; Cowan & Ing, 1934].

To examine the effects of eserine-like substances on nerve two methods were selected. One of them was to measure the action currents of frog's

sciatic nerve trunks which had been soaked in sodium phosphate buffered Ringer's solution and then suspended in moist oxygen, before and after treatment. The other was to record the myograms given by the nerve-sartorius preparation immersed in bicarbonate-buffered Ringer's solution, before and after treatment of only the nerve. The possible alternative of examining the action currents of nerves that had been soaked in bicarbonate Ringer's solution and then suspended in moist oxygen-carbon dioxide mixture was rejected because the rather variable prolongation of the descending phase of the action potential produced by carbon dioxide [Waller, 1896; Cooper, 1924; Davis *et al.* 1928; Gerard & Necheles, 1930] would probably have made it difficult to be certain whether eserine-like compounds produced any further changes.

During the progress of this work a number of papers germane to it have been published. Only two need be mentioned now, the others will be discussed later. Dale *et al.* [1936] found that when the muscles of the frog's hindlimbs were perfused with Ringer's solution containing eserine, stimulation of the ventral nerve roots caused a substance identifiable, pharmacologically at least, as ACh. to appear in the perfusate. They found also that curarine in concentration sufficient to render motor nerve impulses ineffective did not inhibit the liberation of this substance during stimulation, thus rendering more precise Brinkman & Ruiter's earlier observation. Easson & Stedman [1936] have shown prostigmine to be a very effective inhibitor of choline esterase.

I am indebted to Dr E. Stedman for his kindness in allowing me to quote here some of his figures for the inhibitory actions, upon choline esterase, of the same eserine-like substances as were used in my experiments. The figures are part of an extended study which he will publish at some later time.

Materials

The frogs were English *R. temporaria* obtained in fresh batches at about monthly intervals. Except where the contrary is stated the animals had been kept for 3 days before use at a temperature which lay between 14 and 18° C. for the greater part of the time, although the extremes of 12 and 21° C. were sometimes reached. The composition of a bicarbonate-buffered, Ringer's solution which contained 0·1 p.c. of glucose and which would have a *p*H of 7·2 when at 15° C. and in equilibrium with 5 p.c. carbon dioxide mixture was calculated with the help of the Henderson-Hasselbalch equation and Fox's data [1909] for the solubility of carbon

dioxide in saline solutions. In order that the solution should be isotonic with frog muscle [Hill & Kupalov, 1930] the usual sodium chloride content was reduced by 0·016 *M*—an amount corresponding to the added sodium bicarbonate. To avoid the precipitation of calcium carbonate, which would otherwise have occurred when the solution was not in equilibrium with 5 p.c. carbon dioxide mixture, the calcium chloride was made up in a separate and concentrated solution, and before each experiment the required amount was added to a measured volume of the bicarbonate solution which had previously been saturated with oxygen-carbon dioxide mixture. Thus, 5 c.c. of solution containing 4 g. $CaCl_2$ per 100 c.c. were added to 995 c.c. of solution containing 6·0 g. NaCl, 1·34 g. $NaHCO_3$, 0·10 g. KCl, and 1·0 g. glucose. Phosphate Ringer's solution was prepared by adding 10 c.c. of 0·345 *M* sodium phosphate buffer of pH 7·2 to 1 l. of the following solution: 6·75 g. NaCl, 0·10 g. KCl, 0·20 g. $CaCl_2$, and water to 1 l. "AnalaR" chemicals and water distilled in porcelain stills were used for all the solutions.

I am indebted to Roche Products Ltd., London, and F. Hoffmann-La Roche and Co. Ltd., Basle, for supplies of the following eserine-like compounds: prostigmine (the dimethylcarbamic ester of 3-hydroxyphenyltrimethylammonium methylsulphate), the phenylmethylcarbamic ester of 3-hydroxyphenyltrimethylammonium methylsulphate (hereafter Substance 36), the dimethylcarbamic ester of 8-hydroxyquinoline hydrochloride (hereafter Substance 37), the dimethylcarbamic ester of 8-hydroxymethylquinolinium methylsulphate (hereafter Substance 38), 3-hydroxyphenyltrimethylammonium bromide (hereafter Preparation 1210/1), the dimethylcarbamic ester of 3-hydroxyphenylmethyldiethylammonium iodide (hereafter Preparation 3393). Aeschlimann & Reinert [1931] found that the Substances 36, 37, and 38 had certain eserine-like actions, but they did not investigate their actions on muscle, nerve, or neuro-muscular transmission. Preparation 3393 was specially synthesized for the experiments reported in this and a subsequent paper. Solutions of the foregoing compounds or of eserine were made up freshly, in either bicarbonate or phosphate Ringer solution, before each experiment and diluted further as required.

Two specimens of purified curarine chloride were used. They were portions of the preparations which Prof. C. Lovatt Evans and Dr H. King had been kind enough to give to Dr Ing and myself, and which we had used in other work [Cowan & Ing, 1934, 1935]. In preparing the solutions used in the experiments to be described here the molecular weight of curarine chloride was assumed to be 332·7, the value corre-

sponding to Boehm's [1897] formula $C_{19}H_{26}ON_2Cl$. I am indebted to Dr H. R. Ing for a sample of purified phenyltrimethylammonium iodide. Purified tetramethylammonium iodide was prepared by recrystallization from alcohol.

Methods

Nerve action current

The sciatic nerve trunks from a frog weighing 40–60 g. were dissected and allowed to soak for an hour in phosphate-buffered Ringer's solution. At the end of that time one of the nerves was mounted in a paraffin wax chamber, so that the peripheral portion lay on two filter-paper strips connected to two calomel electrodes through which the action current could be led off, and the central end on a pair of platinum wire electrodes through which stimuli could be applied. The peripheral end was crushed and a drop of potassium chloride solution isotonic with frog blood applied to it, in order to secure a monophasic response. Then the chamber was closed with a glass cover sealed on with plasticine, and a slow stream of moist oxygen started. The calomel electrodes were connected in series with a potentiometer, and, through a variable shunt, to a galvanometer which, when critically damped, had a sensitivity 1 mm. $= 2{\cdot}4 \times 10^{-10}$ amp. at 5 m. scale distance, and a period of 3·8 sec. This arrangement permitted the nerve injury potential to be balanced out and the full sensitivity of the galvanometer to be used for observation of the action current when required. Stimulation was by alternate charge and discharge of a condenser, various frequencies being obtained by a rotating commutator which could be driven at any desired speed [Hill, 1934]. The condenser, whose capacity could be varied in steps, was shunted by a resistance of 1500 ohms: a resistance of 4500 ohms was connected in series with the stimulating electrodes. In each experiment the voltage to which the condenser was charged was adjusted until the shocks were slightly supramaximal. A platinum-wire electrode interposed between the stimulating and leading off electrodes, and connected to earth, prevented stimulus escape into the galvanometer circuit. Test periods of stimulation lasting from 3 to 30 sec. and separated by 1 min. intervals were given in groups of six in order that allowance might be made for the initial treppe which occurs in the electrical response of nerve [Waller, 1896; Gerard, 1927]. After the responses to several groups had been observed, the deflexion produced by unbalancing the injury potential by 1–2 mV. was measured, to determine the sensitivity with the nerve included in the circuit. Then, in some experiments the nerve was taken out, immersed

for a time in a solution of an eserine-like substance, remounted, and a fresh injury made; in other experiments the nerve was left undisturbed on the electrodes and was merely painted over with the solution. After treatment the observations of response to test stimuli, together with the determination of sensitivity were repeated several times at half-hourly intervals. A control experiment was made with the second nerve. It was mounted in a duplicate paraffin wax chamber, which could also be connected to the galvanometer and stimulating circuits, and treated in the same way as the experimental nerve except that Ringer's solution was used instead of the solution containing an eserine-like compound.

For experiments with nerve-muscle preparations

A single sartorius with its nerve and the acetabulum was dissected from a frog weighing 20–50 g., and soaked for 1 hr. in bicarbonate-buffered Ringer's solution at 14–16° C. The preparation was then

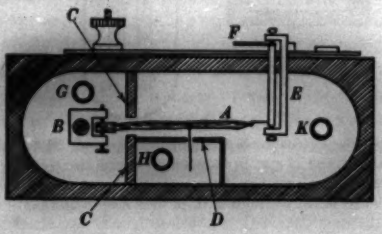


Fig. 1. Modified Lucas trough for experiments with nerve-sartorius preparation. A, preparation in position. B, pin and holder for clamping the acetabulum. C, ebonite partition with shaped hole through which the muscle passes. D, ebonite partition with hole through which the nerve passes. E, Lucas lever crank. F, steel wire connected to isometric lever. G, H, and K, zinc-zinc sulphate non-polarizable electrodes.

mounted in a modified Lucas trough which was filled with the Ringer's solution and in which partitions had been arranged to form fluid electrodes whereby localized stimuli could be applied to the pelvic end of the muscle or to the nerve (Fig. 1). The pelvic end was secured by transfixing the acetabulum with a pin which could be held in a clamp, and the tibial tendon was tied to a Lucas lever crank, the other end of which was connected, by a steel wire, to an isometric spring torsion lever. The period of the moving system, when unconnected to a muscle and when the lever was writing on a smoked paper was about $\frac{1}{10}$ sec. Usually the initial tension to which a muscle was subjected was 2–5 g.; the maximum

amount of shortening permitted to a muscle of 30 mm. length was about 1 mm. In each of the compartments of the trough a separate jet supplied the carbon dioxide-oxygen mixture which kept the bath both stirred and at the correct *p*H. In the compartment containing the muscle and the lever crank a thin-walled glass tube through which cold water could be passed served to maintain the temperature at $15 \pm 0{\cdot}5°$ C. Current could be conducted to each compartment of the trough by zinc-zinc sulphate non-polarizable electrodes. These were of the pattern devised by Lucas [1906], but modified so that any zinc salt which might leak from the filter candle would be unlikely to reach the preparation (Fig. 2). The

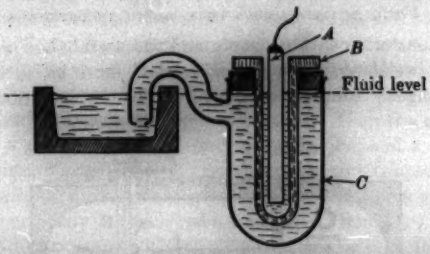


Fig. 2. Non-polarizable electrodes. A, amalgamated zinc rod, 10 mm. diameter. B, filter candle 90 mm. long, and 24 mm. in diameter filled with zinc sulphate solution. C, outer vessel filled with Ringer's solution. To prevent siphoning, the electrodes were arranged so that the menisci of the solutions in them were level with that of the solution in the muscle trough.

relatively large current required for stimulation of the pelvic end of the sartorius, and the loss of part of this current, by the shunting which is inevitable with fluid electrodes of the type used, necessitated large non-polarizable electrodes. They were of the dimensions shown in Fig. 2. Mr C. M. Fletcher, who was good enough to examine the electrodes with the amplifier and oscillograph described by him in a recent paper [1937], found that they were capable of taking 10 mA.—a current considerably greater than was used in my experiments—without appreciable polarization, provided that the zinc rods had been thoroughly amalgamated, but that when the zinc-mercury surface became tarnished polarization developed even with small currents. Although he also found that properly prepared electrodes would last for several days without noticeable deterioration, I preferred to be on the safe side and prepare them freshly each day. Stimulation at various frequencies was effected by condenser

and rotating commutator. The current for the nerve was led in through the electrodes *K* and *H* (Fig. 1), that for the muscle through the electrodes *G* and *K*. For the stimulation of the nerve the condenser used was shunted by a resistance of 1500 ohms, and a resistance of 4500 ohms was connected in series with the nerve electrodes. The resistance between the nerve electrodes was measured in a number of experiments and found to be 4500 ± 100 ohms. Hence the effective resistance shunting the "nerve" condenser was about 1090 ohms. For stimulation of muscle the shunt resistance was omitted since otherwise the voltage to which the condenser would have had to have been charged to elicit maximal response would have been inconveniently large. The resistance between the muscle electrodes was also measured in a number of experiments and found to be 4400 ± 100 ohms, whence the effective resistance shunting the "muscle" condenser was 5900 ± 100 ohms. The duration of stimulation was hand-controlled by a short-circuiting key. A second key beside the first operated a magnetic signal which indicated approximately beneath the myograms the beginning and ending of stimulation. When it was desired to apply single test shocks, or shocks at very low frequency, the short-circuiting key was opened and, by means of a switch, the commutator was replaced by a hand-operated morse key with three contacts. After each preparation had been mounted it was allowed to soak for a further half hour, then the bath was drained and refilled with fresh Ringer's solution before beginning an experiment.

Essentially the procedure in these experiments was to stimulate the nerve or the muscle of the preparation for test periods, each lasting several seconds, at regular intervals. In some experiments the nerve and the muscle were stimulated alternately; in others the muscle was stimulated only once for every three or every six times that the nerve was stimulated. Each experiment was begun by making a sufficient number of records to establish that stimulation of the muscle and of the nerve elicited reproducible responses. When this had been done, the trough was emptied, refilled with the solution whose action was to be investigated, and further test stimuli applied.

For the experiments in which it was intended to immerse only the nerve in a solution of an eserine-like substance and the remainder of the preparation in Ringer's solution the Lucas trough was modified further as shown in Fig. 3. An additional partition was placed within the small compartment which contained the greater part of the nerve and the central end of the nerve was passed through a hole in this partition. The two small compartments could be filled with the solution

whose action was to be examined and, by leading current into them through non-polarizable electrodes, the nerve could be stimulated within the region that had undergone treatment. To prevent any eserine-like substance reaching the muscle and nerve endings the hole in the outer of the two partitions through which the nerve passed was blocked with vaseline, and the Ringer's fluid in the two larger compartments which contained the muscle was changed frequently during an experiment. Except for these modifications the arrangements for stimulation were

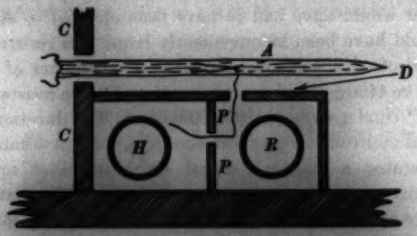


Fig. 3. The Lucas trough further modified for the experiments in which only the nerve was treated with a solution of an eserine-like substance. The two small compartments in which the nerve lay were filled with the solution. The letters A, C, D, and H have the same meaning as in Fig. 2. P, additional partition with hole through which the nerve passed. R, zinc-zinc sulphate non-polarizable electrode.

those described in the previous paragraph but one. In several experiments the resistance between the nerve electrodes was found to be 4400 ± 100 ohms, hence the effective resistance shunting the condenser was about 1120 ohms. In each experiment preliminary records of the responses to stimulation of muscle and of nerve were made whilst all the compartments were filled with Ringer's solution, then the two smaller compartments were drained, filled with the solution whose action was to be examined and responses to further stimuli were recorded.

Results

Nerve action current

Using the full sensitivity of the galvanometer the deflexion produced by stimulation of Ringer-soaked nerve for periods of 3–30 sec., at frequencies ranging from 5 to 200 per sec., was 10–450 mm. In most experiments however the galvanometer shunt was adjusted so that the maximum deflexion did not exceed 150 mm., since it was found that

deflexions of this order could be read to within about 1 p.c. and that the increased accuracy which might have been expected with larger deflexions was rather more than offset by small changes of zero, probably owing to elastic hysteresis of the metallic strip suspending the galvanometer coil, and by the difficulty of making readings when the light spot was moving rapidly.

In a preliminary communication [1936*a*] I stated that treatment of nerve with prostigmine in concentrations up to 1000 μM (1000×10^{-6} g.-mol./l.) for 4 hr. produced no change in the action current. After further experiments I think that this statement may have to be modified slightly, but I am not yet quite certain.

Treatment of nerve with solutions of 1, 10, or 100 μM eserine, prostigmine, or Substance 38, for periods up to 3 hr., caused no greater change in the responses to stimulation at the frequencies and for the periods mentioned than did treatment with Ringer's solution (about 3–10 p.c. diminution). Treatment for longer times, up to 6 hr., also caused no indisputable change in the responses. In these observations the experimental uncertainty was generally greater than in those lasting for the shorter time because of the greater differences between the decline of the responses of different experimental and of different control nerves (3–15 p.c.). The soaking of nerve for 3–6 hr. in a solution containing 1000 μM prostigmine caused the response to increase by 10–20 p.c. in some but not in all experiments. In those experiments in which the increase appeared, and in those in which it did not appear, the movement of the galvanometer spot showed no sign of irregularity or temporary failure, either with high or low frequency stimulation.

Nerve-muscle preparations which had been soaked in bicarbonate Ringer's solution

Responses to stimulation of muscle.

Preliminary experiments led me, for reasons which will be clear from the results described under the next subheading, to employ test periods of stimulation lasting 10–30 sec. Maximal stimulation with 15–30 shocks per sec. elicited responses in which the initial rise of tension exhibited wavelets due to incomplete summation to an extent dependent upon the frequency used. These became smaller and disappeared within 1–3 sec. and the rise continued for 1–4 sec. longer, this time also depending mainly upon the frequency used. After passing through a blunt maximum the tension then declined very slowly. Except that the rate of fall

following the maximum is much too fast Fig. 10H illustrates the type of response obtained. Stimulation with 50–120 shocks per sec. elicited responses in which the whole or the greater part of the initial rise of

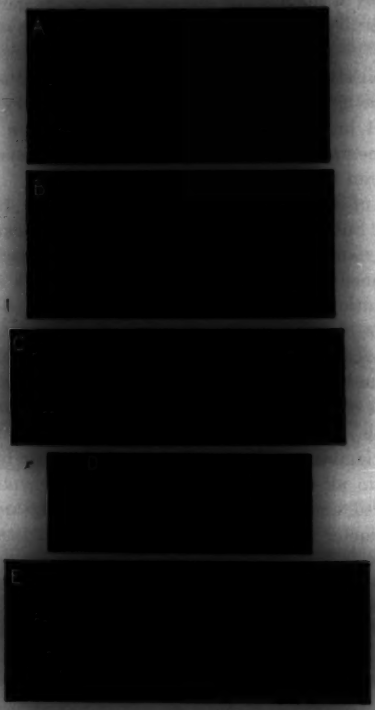


Fig. 4. 26. xi. 35. The effect of 0·03 μM solution of prostigmine upon the responses of a sartorius nerve-muscle preparation to stimulation with 100 shocks per sec. The time markings are seconds. The depression of the signal line at the left of each record indicates the beginning of stimulation, and the rise at the right of each record the end of stimulation. A, 3.32 p.m., nerve of Ringer-soaked preparation stimulated with maximal shocks of time-constant 109 μsec. 3.45 p.m., the Ringer's solution in the bath was replaced by solution containing prostigmine. B, 6.15 p.m., nerve stimulated as in A. C, 6.41 p.m., nerve stimulated as in A. D, 7.11 p.m., nerve stimulated as in A. E, 7.24 p.m., pelvic end of the muscle stimulated with shocks of time-constant 1475 μsec.

tension took place in one step. After remaining steady or increasing very slightly in the next few sec. (4–6) the tension then began to fall slowly (Fig. 4E). Stimulation at higher frequencies, 120–200 shocks per sec., caused the tension to attain its maximum value almost immediately. In the following 5 sec. or so the tension fell very slowly or remained constant; after that, its fall became a little more rapid (Fig. 5A). When the stimulation periods were separated from one another by 10 min. intervals the successive responses of any one muscle to repeated application of the same stimulus usually differed very little from one another. After about 3 hr. of such intermittent activity the maximum tension attained was from 2–10 p.c. lower than in the responses recorded at the beginning of the experiment, and the rate of decline of tension during the responses was also rather greater (generally 5–10 p.c.). Experiments within the breeding season, or the month after, were avoided as far as possible, since the muscle responses deteriorated more rapidly than at other times (cf. Fig. 9 A, E), and the differences between the responses of muscles from different frogs were greater.

The foregoing results were uninfluenced when the time-constant of the shocks comprising the stimulus was varied between 590 and 1475 μsec., and when the voltage to which the condenser was charged was increased to 20 p.c. above the value required to produce maximal response.

During the first few of the above experiments the adequacy of the buffering of the solution in the trough was tested by taking out samples periodically from the muscle compartment and determining their *p*H colorimetrically. Provided that the stream of oxygen-carbon dioxide mixture was sufficiently fast the *p*H found initially was $7{\cdot}2 \pm 0{\cdot}05$—the figure calculated. After 100 min., in which the muscle had undergone ten test periods of activity, a diminution of about 0·1 *p*H unit was found. In subsequent experiments (with or without drugs) the solution in the trough was renewed at least once for every ten test stimuli applied to the muscle or the nerve of the preparation.

Responses to stimulation of nerve.

As the frequency of stimulation was increased from 15 to 200 per sec. the form of the response became progressively modified. It is convenient, however, to make a somewhat arbitary classification into three main types. The first of these was generally elicited at frequencies 15–60 per sec. The maximum tension, which was slightly greater than that obtained by direct stimulation of the muscle at the same frequency as was used for the nerve, was attained in two steps—a rapid initial rise passing over

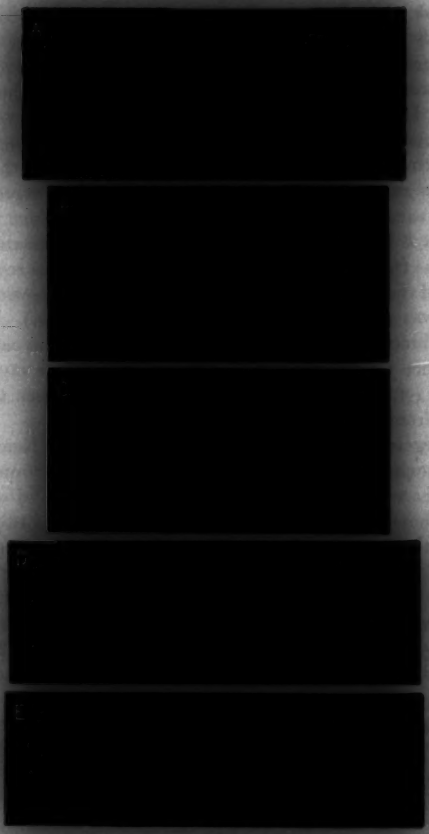


Fig. 5. 21. xi. 35. The effect of 0·3 μM prostigmine solution on the responses of a sartorius nerve-muscle preparation to stimulation with 150 shocks per sec. Time markings and signal line have same meaning as in Fig. 4. A, 4.22 p.m., pelvic end of Ringer-soaked muscle stimulated with maximal shocks of time-constant 1450 μsec. B, 5.22 p.m., nerve stimulated with shocks of time-constant 109 μsec. 5.25 p.m., the Ringer's solution in the bath was replaced by solution containing prostigmine. C, 5.52 p.m., nerve stimulated as in B. D, 6.2 p.m., nerve stimulated as in B. E, 6.33 p.m., nerve stimulated as in B.

into a very slow rise lasting several (3–7) sec. After the maximum had been attained the tension fell at a slow rate which was not much greater than that at which it fell in the corresponding part of the response to direct stimulation of the muscle. With stimulation frequencies 15–30 per sec. the slow part of the rise of tension exhibited wavelets which became smaller and disappeared within a second or two. The second type of response was produced generally at frequencies 100–180 per sec. It was characterized by a secondary rise of tension which appeared several seconds after the first maximum. Stimulation with about 100 shocks per sec. caused the tension to attain its maximum in two steps similar to those mentioned in connexion with the first type of response. Usually the maximum was 1–5 p.c. greater than the tension obtained by direct stimulation of the muscle at the same frequency. The tension began to fall slowly from the maximum, but after two or three seconds there appeared superimposed on the fall a small and slow rise which lasted for a few seconds. The tension passed through a second maximum and then fell slowly again (Fig. 4A). Stimulation at a rate of about 150 shocks per sec. also caused the tension to rise to its maximum in two steps, and again the maximum was slightly greater than was obtained by direct stimulation of the muscle. After about a second the slow part of the initial rise was cut short by a rapid fall lasting for a few seconds (usually 2–6 sec.): this was succeeded by a rather slower (secondary) rise which continued for several seconds and which was afterwards followed by a very slow fall (Figs. 5B, 10A). With a stimulation frequency of about 180 per sec. the initial rise of tension was cut short before it had become fully developed by a rapid fall lasting 2–5 sec.; after passing through a minimum, which was often only 20–30 p.c. of the maximum tension that the muscle yielded in response to direct stimulation at the same frequency, the tension rose again fairly quickly to its secondary maximum and then fell very slowly. The third type of response was elicited at a frequency of about 200 per sec. In it the rapid fall which cut short the initial rise occurred earlier than in the responses of the second type and proceeded until the tension had become very small. On continuing stimulation for a further 15–25 sec. the tension did not rise again or rose very little. Secondary differences between the responses of different preparations were more marked than at lower frequencies. Some preparations yielded a rise and fall of tension which was similar to that exhibited in a single twitch, and which was followed by a very slowly diminishing tension; others yielded a rather greater initial tension followed by a rapid fall and then a very slow rise.

When the nerve of a preparation which had suffered no stimulation for an hour or more was stimulated at 10 min. intervals at a frequency which produced responses of the second or third type, the rate of fall of tension in the early part of the first response was often a little slower than it was in the second response. Subsequent responses differed little. In the responses recorded after about 3 hr. of this periodic activity the initial tension was generally 5–10 p.c. smaller than it had been at the beginning of the experiment; also, the form of the tension-time curve had become modified in such a way as might have been imitated at the beginning of the experiment by using a stimulation frequency slightly higher than was actually used, i.e. in the experimental period the frequency required to produce a particular form of response diminished slightly. In some experiments it was convenient to allow longer rest intervals (up to 30 min.) between the stimulation periods: the changes that occurred in the responses were similar to those just described.

The various forms of response which have been described in this section were obtained equally well when the time-constant of the shocks making up a stimulus was given values ranging from 54·5 to 218 μsec., and when the voltage was varied between 1·0 and 1·5 times that needed to evoke maximal response.

The responses of preparations whose nerves only had been treated with eserine-like compounds

The nerves were immersed for 3–4 hr. in Ringer's solution containing 3–300 μM prostigmine, or 0·03–50 μM eserine, or 3–30 μM Substance 38. During the test period the myograms in response to maximal or slightly supramaximal stimulation of the nerve did not change any more than did those given by the untreated preparations (see the preceding section).

Preparations which had been treated with eserine-like compounds

Responses to stimulation of muscle.

Treatment for 3–4 hr. with a solution containing any of the following compounds within the concentration ranges stated produced no change in the responses of muscle:

Prostigmine, 0·03–3000 μM.
Eserine, 0·03–50 μM.
Substance 36, 0·03–30 μM.
Substance 38, 0·03–30 μM.
Preparation 1210/1, 0·03–50 μM.
Substance 37, 0·03–20 μM.

Substance 37 in concentrations higher than those mentioned above reduced the tension developed by the muscle but did not alter the course of the decline of tension during stimulation at various frequencies. A 400 μM solution reduced the tension by about 30 p.c.

Preparation 3393, used in concentrations 5–53 μM, did not change the form of the response within 3 hr. If, however, the stimuli were reduced to considerably submaximal strength small twitchings similar to those recorded in Fig. 10 C–F appeared superimposed on the response.

Responses to stimulation of nerve.

The principal action of any of the eserine-like substances was to lower the frequency required to evoke the responses which were termed the second and third types in the section dealing with Ringer-soaked preparations; certain other modifications in the form of the response were also produced. For brevity and clearness the action of prostigmine will first be described and afterwards the respects in which the actions of other compounds resembled or differed from that of prostigmine.

The interval which elapsed between filling the bath with solution containing prostigmine and a perceptible change in the response varied according to the concentration used and the frequency of stimulation. The interval also varied with different preparations, presumably on account of differences of sensitivity, a factor which will be discussed later in connexion with the comparison of the activities of the eserine-like substances. When preparations were immersed in a 0·03 μM solution and tested with stimuli of frequency 100–200 per sec. some change could be detected within an hour, but the principal changes took place in the next 1½–2½ hr. The prostigmine caused the form of the response to stimulation with 100 shocks per sec. to change in such a way as might have been imitated by stimulating the untreated preparation with about 150 shocks per sec. (Fig. 4 A–D). Sometimes small irregular variations of tension also appeared (Fig. 4 B, C, and D). Apart from these changes the maximum initial tension developed in the later responses was only one-half to three-quarters of what it would have been had the untreated preparation been stimulated with 150 shocks per sec.

With 0·3 μM prostigmine and a stimulation frequency between 100 and 200 per sec. the latent period for a perceptible change in the response was reduced to 25–35 min., and the principal changes took place in the next ½–1 hr. When the nerve was stimulated with 150 shocks per sec. prostigmine first caused a smaller initial tension to be developed and the minimum to occur earlier and to be smaller than in the responses of

the untreated preparations; also it caused the tension developed in the secondary rise to increase, usually by 5–10 p.c. (cf. Fig. 5 B, C). After the prostigmine had been allowed to act for another 10–20 min. the early part of the response became irregular. Often the tension rose initially to only one-quarter to one-third of the maximum value that the untreated preparation had given, fell momentarily, and then resumed its ascent; after attaining the first maximum it fell in a very irregular manner to its minimum which occurred still earlier than in previous responses: subsequently the tension rose again to a height that was the same or greater than in earlier records and then declined very slowly (Fig. 5 D). In responses recorded between 15 and 30 min. later the tension rose initially to a height that would have been given by one or two maximal shocks applied to the nerve of the untreated preparation and then fell rapidly to its minimum. Having passed through the minimum the tension rose again in the next 10–15 sec. to a value that was usually rather less (5–10 p.c.) than had been given by the untreated preparation, and afterwards fell very slowly (Fig. 5 E).

With 3 μM prostigmine and a stimulation frequency 100–200 per sec. a perceptible change in the response occurred in 10–25 min.: the change continued and became maximal within 60–80 min. When the nerve was tested at intervals, with shocks at the rate of 150 per sec., the response passed through changes very similar to those described in the preceding paragraph and illustrated in Fig. 5; afterwards further changes took place in the part of the response which followed the minimum of tension. The secondary rise became slower and smaller until finally the response comprised an initial tension, such as might have been evoked by one or two maximal shocks applied to the nerve of the untreated preparation, followed immediately by a fall to a very small tension which diminished very slowly during stimulation for a further 20 sec. Except for the superimposed small irregularities, the responses shown in Fig. 10 D–F may be taken as illustrating the later responses obtained after treatment with 3 μM prostigmine solution. When test stimuli of a lower frequency (20–30 shocks per sec.) were applied the tension rose initially to about three-quarters of what had been obtained by stimulating the untreated preparation with 150 shocks per sec. The tension was maintained for about a second, then it fell to one-half or one-third of its initial value in 4–6 sec. and afterwards more slowly. Except for the superimposed irregularities Fig. 10 G illustrates the type of response obtained. When test stimuli of still lower frequency (about 15 per sec.) were used the tension rose relatively slowly and showed wavelets due to incomplete

summation. These disappeared after 1–2 sec. and the rise continued for another 2–3 sec. The tension passed through a maximum which was about two-thirds of that attained in the response of the untreated preparation to stimulation with 150 shocks per sec., and afterwards fell at a rate which although slow was considerably greater than in the response of the untreated preparation to stimulation at the same frequency. Fig. 10H illustrates the type of response obtained.

Prostigmine in still greater concentrations reduced further the latent period for a perceptible change in the response to stimulation with 100–200 shocks per sec.; it became much the same, 5–10 min., with a concentration of 300 or 3000 μM. The response to stimulation with 150 shocks per sec. soon came to follow the course described above: initial rise, rapid fall to small tension, which afterwards diminished very slowly. In subsequent responses the initial rise and rapid fall of tension suffered no further change whereas the very slowly diminishing tension which followed became larger, and often was better maintained than in the responses of preparations that had been treated with prostigmine in smaller concentrations. Upon stopping stimulation which had been going on for about 20 sec. the persistent low tension fell very rapidly to one-half to one-third of its previous value, and then the remaining tension gradually disappeared in several seconds (often 5–10). Presumably it was due to contracture. The responses of preparations that had been treated with prostigmine in the higher concentrations, to low frequency (30–60 shocks per sec.) stimulation, differed from the responses to stimulation at higher frequencies only in that the tension which followed the initial rise and fall was smaller and disappeared more quickly when stimulation was stopped.

In two experiments a preparation was immersed in a 3000 μM solution of prostigmine, and the responses to stimulation of the nerve and to direct stimulation of the muscle, at frequencies of 100 and 150 per sec., were recorded several times during the first hour; then the preparation was left overnight and on the following day, 14–16 hr. later, the responses to stimulation of the nerve and of the muscle were again recorded. A control preparation which was immersed only in bicarbonate Ringer's solution was left overnight and similarly tested. After 14–16 hr. the responses of the prostigmine-treated and of the control muscles to direct stimulation at the higher frequency differed very little from one another; the responses at the lower frequency also were very similar to one another. In both cases they were of the same form as those obtained on the previous day, but the maximum initial tension was about 20 p.c.

smaller, and the rate of decline of tension during response was slightly greater. After 14–16 hr. the prostigmine-treated preparations gave responses to nerve stimulation very similar to those that they had given after treatment for about an hour except that the maximum initial tension had diminished by about 30 p.c. The responses of the Ringer-soaked preparations to nerve stimulation also differed little from those obtained on the previous day, except that the tension developed had diminished by about 30 p.c.

The various forms of response which have been described under this sub-heading were uninfluenced when the time-constant of the shocks making up a stimulus was varied between 54·5 and 218 μsec., also when the voltage was increased to 1·5 times that needed to evoke maximal response.

A few experiments were made with preparations from frogs which had been stored at 0–5° C. for 40 hr. or more before use ("cold" frogs). The dissection was done at room temperature and as soon as it was complete the preparation was mounted in bicarbonate and glucose Ringer's solution in the modified Lucas trough at 15° C. After ½ hr. the experiment was made in the way already described. The responses to maximal stimulation of the pelvic end of the muscle differed little from those given by the "warm" frog preparations: at low frequencies (20–30 per sec.) during the initial rise of tension the wavelets due to incomplete summation were rather less marked; at higher frequencies (100–150 per sec.) the rate of decline of tension was generally a little greater. The form of the response to maximal stimulation of the nerve was less sensitive to changes of frequency than it was with "warm" frog preparations. When shocks of time-constant 54·5 or 109 μsec. were applied at frequencies between 20 and 30 per sec. the wavelets which occurred during the initial rise were again less marked. At stimulation frequencies between 120 and 160 per sec. the maximum tension attained in the initial rise was about 10 p.c. higher than was obtained by stimulation of the pelvic end of the muscle; the minimum to which the tension afterwards sank was relatively less deep than in the responses of the "warm" frog preparations and the maximum tension attained in the secondary rise was greater (Fig. 6A, B). The form of the response to stimulation at any one frequency did not remain constant however. The longer the "cold" frog preparations were soaked in the Ringer's solution the more did their responses come to resemble those of the "warm" frog preparations. The soaking of "cold" frog preparations for 1½–2 hr. sufficed to render their responses to stimulation with 150 shocks per sec. indistinguishable from those of "warm" frog preparations (cf. Fig. 6B, C).

The responses of prostigmine-treated "cold" frog preparations differed consistently from those of prostigmine-treated "warm" frog preparations in certain respects. The principal differences were that at low frequencies prostigmine caused the maximum tension developed in the response to occur earlier and to become greater; that at high frequencies prostigmine did not cause the tension developed in the initial rise to be reduced to the same degree that it did in the responses of the "warm" frog preparations. When "cold" frog preparations which had had little soaking in Ringer's solution were treated with prostigmine these differences largely persisted for several hours (more than 4). Also, although soaking for about 2 hr. in Ringer's solution abolished the differences between the responses of "cold" and "warm" frog preparations it did not prevent the differences

between prostigmine-treated "cold" and "warm" frog preparations from appearing. In the next two paragraphs the differences will be described in more detail.

In the response to stimulation at a frequency of about 150 per sec., about 15 min. after filling the bath with 3 μM prostigmine solution, there appeared a small augmentation (usually 3–5 p.c.) of the tension developed in the initial rise; also, a marked deepening

Fig. 6. 15. iii. 38. The effect of soaking in Ringer's solution followed by treatment with prostigmine upon the responses of a nerve-sartorius preparation from a "cold" frog. Time markings and signal line have the same meaning as in Fig. 4. Stimulation frequency 150 per sec., except in F. 1.50 p.m., dissection finished. 1.55 p.m., preparation mounted in bath. A, 2.38 p.m., pelvic end of muscle stimulated with shocks of time-constant 1475 μsec. B, 2.48 p.m., nerve stimulated with shocks of time-constant 73 μsec. C, 3.36 p.m., stimulation of nerve repeated. 3.44 p.m., bath drained and filled with solution containing 3 μM prostigmine. D, 4.20 p.m., stimulation of nerve repeated. 4.22 p.m., bath drained and filled with solution containing 12 μM prostigmine. E, 5.2 p.m., stimulation of nerve repeated. F, 6.0 p.m., nerve stimulated with 50 shocks per sec.

of the subsequent minimum (Fig. 6D). What happened to the tension developed in the secondary rise depended on how long the preparation had been soaking in Ringer's solution. If the soaking had been only for a short time then the reduction produced by the Ringer's solution generally outweighed the augmentation produced by the prostigmine; if, on the other hand, the soaking had been sufficiently long for the preparation to reach a fairly steady state then augmentation resulted (Fig. 6D). After another $\frac{1}{2}$ hr., or a shorter time

If a more concentrated prostigmine solution were used, the initial maximum became slightly reduced, the fall which followed the maximum became earlier and faster, and the secondary rise was depressed. Still later the initial maximum was reduced to about two-thirds of what had been obtained at the beginning of the experiment: as soon as the maximum had been attained the tension fell rapidly to one-quarter to one-third of its initial value and afterwards slowly at a diminishing rate (Fig. 6E). Stimulation of a preparation when in this condition at a frequency of 50 per sec. elicited a response of similar form to that just described, but of greater tension throughout (Fig. 6F).

Treatment of a preparation for about ½ hr. with 6 μM prostigmine solution caused the maximum tension developed during the response to stimulation at a frequency of 20 per sec. to be reached by a rapid initial rise instead of by a rapid rise followed by a slow rise. Once the tension had reached its maximum it was better maintained during 15–20 sec. of continued stimulation than it had been before the treatment (cf. Fig. 7A, B). After a further

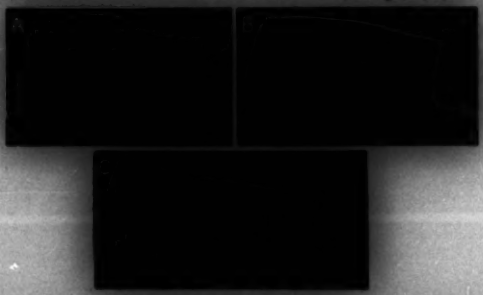


Fig. 7. 18. iii. 38. The action of 6 μM prostigmine solution upon the responses of a nerve-sartorius preparation from a "cold" frog to stimulation at a frequency of 19 per sec. The time constant of each shock was 73 μsec. Time markings and signal line have the same meaning as in Fig. 4. 2.0 p.m., dissection completed. 2.5 p.m., preparation mounted in bath of Ringer's solution. A, 3.30 p.m., nerve stimulated. 3.33 p.m., bath drained and filled with solution containing 6 μM prostigmine. B, 3.57 p.m., nerve stimulated. C, 4.51 p.m., nerve stimulated.

1–1½ hr. in 6 μM prostigmine solution the maximum tension in the response was still developed in the initial rise and usually had become increased by about 5 p.c. although the response of the muscle to direct stimulation had diminished by about that amount. After the maximum had been reached the tension fell more rapidly than in earlier responses (Fig. 7C).

Apart from producing changes in the form of response prostigmine had two other actions. The first was to produce a few "spontaneous" twitches of the "cold" frog preparations which had been soaked for only a short time in Ringer's solution, but not of the preparations which had been soaked for an hour or more. Usually the twitches appeared about 10 min. after filling the bath with prostigmine solution, continued at irregular intervals for 2–3 min. and then disappeared. The second action was to cause each period of high frequency stimulation to be followed 1–4 min. later by one or more "spontaneous" twitches (Fig. 6D, E, F). The twitches were obtained with preparations which had been soaked in Ringer's solution for short or for long times. They were not observed after stimulation at frequencies of 20–30 per sec.

In three experiments the action of 3000 μM prostigmine solution upon the responses of preparations to slow rhythmic stimulation was examined. Two of the experiments were with preparations from "warm" frogs, and one was with a preparation from a "cold" frog. After the preliminary soaking each experiment was begun with the recording of two series of responses: one to direct stimulation of the muscle by maximal shocks of time constant 1475 μsec. at the rate of about 5 per min., the other to stimulation of the nerve by maximal shocks of time constant 109 μsec. at the same rate. The bath was then drained and filled with the prostigmine solution. Rhythmic stimulation was begun again 10 min. later. In the first few responses given by the "warm" preparation the maximum tension was unaltered; in the next few responses it declined about 20 p.c. and then recovered slowly to its initial value as stimulation continued. During rhythmic activity for another $\frac{1}{2}$ hr. the maximum tension developed in each response remained fairly constant—at the end of that time it was about 95 p.c. of the initial (untreated) value. The preparation was then left undisturbed overnight and tested again 14–16 hr. later, with two more series of rhythmic shocks, the first being applied to the muscle and the second to the nerve. After the initial treppe, the muscle response became fairly steady at about 80 p.c. of the previous day's value. When the nerve was stimulated the maximum tension developed in the first response was about 70 p.c. of the previous day's initial value for the untreated preparation; in subsequent responses the maximum tension declined to about 50 p.c. and afterwards recovered more slowly than it had done on the previous day. In the experiment with the "cold" preparation, on recommencing stimulation of the nerve at the rate of about 4 shocks per min., 10 min. after the bath had been filled with the prostigmine solution, the maximum tension developed in the first few responses was about 20 p.c. greater than had been given by the untreated preparation (cf. Fig. 8A, B). In the next few responses the maximum tension declined until it exceeded the initial (untreated) value by only 5 p.c., and then, as stimulation continued, it recovered slowly until it exceeded the initial value by about 10 p.c. After the recovery the rhythm was reduced to about 3 shocks per min., without omitting any shocks, and stimulation was continued for another $\frac{1}{2}$ hr. During that period the maximum tension developed in the responses remained fairly steady, and at the end of the period it was still about 5 p.c. greater than that given by the untreated preparation. The preparation was left overnight and tested on the following day with stimuli at the rate of 4 per min. The maximum tension developed in the response of the muscle to direct

stimulation had diminished to 85 p.c. of the previous day's value. The maximum tension developed in response to stimulation of the nerve was 77 p.c. of what had been given by the untreated preparation on the previous day. In subsequent responses the maximum tension diminished to 51 p.c., and afterwards made a slow and incomplete recovery. In two control experiments, in which untreated preparations were left mounted overnight, as in the experiments with prostigmine, the maximum tension evoked by stimulation of the nerve diminished rather more than did the maximum tension evoked by direct stimulation of the muscle.

Fig. 8. 1. viii. 35. The action of a solution containing 3000 μM prostigmine upon the responses of a sartorius nerve-muscle preparation from a "cold" frog. Time markings at 5 sec. intervals. Each response was elicited by a single maximal shock to the nerve. In order that the changes may be more easily seen a dotted line has been drawn parallel to the resting tension line and at a height which represents the maximum tension attained in the responses before the preparation was treated with prostigmine. 6.0 p.m., preparation mounted in Ringer's solution. 8.55 p.m., fresh Ringer's solution in bath. A, 9.12 p.m., the steady value of the response, before treatment. 9.14 p.m., bath drained and then filled with prostigmine solution. B, 9.24 p.m., stimulation recommenced. C, responses recorded at 9.55 p.m. and after.

By the application of eserine, Substance 36, Substance 38, or Preparation 1210/1, in suitable concentration to a nerve-sartorius preparation it was possible to produce changes in the response to nerve stimulation closely similar to those produced by prostigmine in concentrations between 0·03 and 30 μM.

Since it was necessary to allow some considerable time to elapse after the treatment of a preparation with an eserine-like substance, and during that time to record the responses to several test stimuli, in order to ascertain if the action of the substance had become fully developed, I thought it better not to try to compare quantitatively the actions of different solutions of prostigmine, or of prostigmine and another eserine-like substance, on one preparation, but instead to take a fresh preparation for each solution. It has been pointed out already that the successive

responses of a Ringer-soaked preparation to stimulation at one frequency differed little from one another, but that the responses of different preparations to stimulation at the same frequency exhibited minor differences. Before making the comparison of the activities of the eserine-like substances with that of prostigmine it was necessary to obtain some estimate of how much uncertainty would be introduced by these differences of response and the differences of sensitivity exhibited by different preparations towards any one of the eserine-like substances. The following method was used to obtain the estimate. Ringer-soaked nerve-sartorius preparations of about the same size were taken, two at a time, and after their responses to stimulation for 15 sec. periods at a rate of 100 or 150 shocks per sec. had been recorded one of them was treated with a 0·1–0·5 μM prostigmine solution and the other was immersed in a prostigmine solution twice as concentrated as the first; subsequently the changes which occurred in the responses of both preparations to further test stimuli were followed. These conditions were selected partly because they were ones in which changes of response were well marked, and partly because, as will be seen later, they were ones in which prostigmine reversed, in some measure, paralysis due to curare-like substances. Thirteen experiments were made. The records showed that it would be impossible to take the change produced in any single feature of the response as a measure of the concentration of the prostigmine solution with which the preparation had been treated. Instead the changes produced in complete responses were compared. In seven of the experiments it was possible to distinguish between the higher and lower concentrations of prostigmine, in three of them it was impossible, and in three distinction was doubtful.

After the experiments with prostigmine had been made, the concentration was determined of each of the other eserine-like substances that would produce changes in the responses to stimulation at a frequency of 100 or 150 shocks per sec., which matched as nearly as possible those produced by 1 μM prostigmine solution. The results are given in Table I, and with them Dr Stedman's figures for the choline-esterase-inhibiting activities of the same compounds.

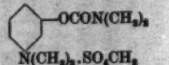
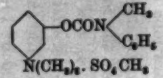
The relatively great activity of Preparation 1210/1 on neuro-muscular transmission (e.g. Fig. 9A–C) was rather unexpected because this compound contains no urethane grouping, and further experiments were made to check the result. Dr Ing was kind enough to recrystallize a specimen of Preparation 1210/1 for me. After recrystallization its activity was the same as before, within the limits of the experimental

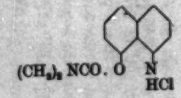
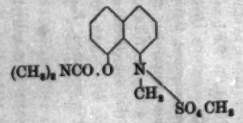
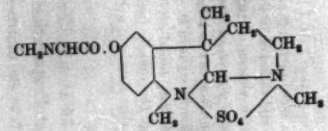
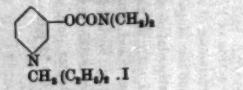
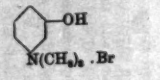
TABLE I

Co 4 shows concentration necessary to produce changes in the response to nerve stimulation, with 100 shocks per sec., closely similar to the changes produced by 1 μM prostigmine solution.

Col. 6 shows extent to which eserine-like compounds restored the responses of partly curarized preparations to nerve stimulation with 100 shocks per sec. Each experiment is summarized by the ratio of maximum tension developed after treatment with the eserine-like compound to the tension whilst the preparation was under the influence of curarine alone. Both tensions are stated as percentages of the maximum before the treatment with curarine chloride.

Col. 7 shows experiments the same as those in the preceding column except that phenyltrimethylammonium iodide was used instead of curarine chloride to produce partial paralysis.

(1)	(2)	(3)	(4)	(5) *Stedman's figures.* Percentage inhibition of choline-esterase produced by each compound in concentration 5×10^{-4} M, at pH 7·4, after		(6)	(7)
	Chemical structure	Mol. wt.	In μM	1 hr.	4½ hr.		
Prostigmine		334·0	—	35	72	40/8 42/11 20/8	23/14 38/9
Substance 36		396·0	5 2 2	Mean = 3·0	15	30	44/9 31/6 27/7	33/10 42/11

Substance 37 	254·5	400 300 500 450	Mean = 413	6	11	21/12 15/10 12/8	22/14 37/16
Substance 38 	344·0	1·5 1·0 0·75	Mean = 1·1	51	81	61/8 41/16 33/14	71/16 20/8
Eserine sulphate 	373·0	3 6 3 6	Mean = 4·5	58	53	70/13 42/11 53/9	39/12 48/11
Preparation 3393 	378·0	0·5 0·75 1·2	Mean = 0·82	As above but in concentration $2·5 \times 10^{-4}$ *M* after 3 hr.		66/14 52/11 29/8	43/13 64/12
				—	65		
Preparation 1210/1 	232·0	25 25 20 40	Mean = 27·5	As above but in concentration 5×10^{-4} *M* after		23/11 48/9 47/14	46/13 31/16
				3 hr.	21 hr.		
				4	23		

method. Part of the recrystallized specimen was microanalysed by Dr G. Weiler and Dr F. B. Strauss. The figures that they returned: H = 6·03 p.c., C = 46·80 p.c. are very close to the calculated ones: H = 6·08 p.c., C = 46·50 p.c. My own analysis of another part of the recrystallized specimen for bromide gave 34·4 p.c. Br′, which also agrees

Fig. 9. 7. iv. 37. The action of Preparation 1210/1 followed by phenyltrimethylammonium iodide upon the responses of a nerve-sartorius preparation to stimulation at a frequency of 89 per sec. Time markings and signal as in Fig. 4. A, 2.6 p.m., pelvic end of Ringer-soaked muscle stimulated with shocks of time-constant 1500 μsec. B, 2.18 p.m., nerve stimulated with maximal shocks of time-constant 109 μsec. 2.45 p.m., bath drained and then filled with Ringer's solution containing 25 μ*M* of Preparation 1210/1. C, 4.24 p.m., nerve stimulated as in B. 4.26 p.m., 0·14 c.c. of 50 m*M* phenyltrimethylammonium iodide solution added to the compartment of the bath which contained the greater part of the muscle (vol. 25 c.c.) to make the concentration there 280 μ*M*. D, 4.52 p.m., stimulation of nerve repeated. E, 5.24 p.m., stimulation of muscle as in A.

with the calculated value 34·43 p.c. From the additional results and the analyses I conclude that Preparation 1210/1 was not contaminated with an impurity highly active on neuro-muscular transmission.

Estimation of the activities of Substance 37 and of Preparation 3393 was rather more difficult because their actions differed in certain respects from that of prostigmine. After the action of 0·03–0·3 μ*M* Preparation 3393 had become well developed, stimulation of the nerve with 150 shocks per sec. resulted in the tension attaining a greater value initially and

then falling to a less extent than it did after treatment with equimolar prostigmine solutions. The secondary rise was about the same as in the responses of prostigmine-treated preparations, or a little less marked. Small and irregular variations of tension appeared superimposed on the secondary rise, and to a greater extent on the subsequent slow fall. The responses of preparations that had been treated with Preparation 3393 in higher concentrations (3–10 μM) passed through the changes indicated above, then both the maximum initial tension and the secondary rise diminished (Fig. 10A–D). Soon the response came to consist of an initial rise of tension, followed by a rapid fall to a small value, and afterwards by a very slow fall (Fig. 10E). Generally, the small and very slowly falling tension was free from irregularities for several seconds after the rapid fall; later, however, rapid and irregular variations appeared and they increased in amplitude as stimulation continued. On examining the muscles of preparations during these responses it could be seen that the rapid and irregular variations were due to twitchings of small groups of fibres. In subsequent responses the initial tension diminished further, but very often it exceeded that developed in the responses of preparations which had been treated with the corresponding amount of prostigmine; also, the rapid and irregular variations which appeared in the later parts of the responses became less marked (Fig. 10F). When a test stimulus of frequency about 25 shocks per sec. was employed the tension rose to about three-quarters of the maximum that the untreated preparation had given on stimulation with 150 shocks per sec., fell in a few seconds to about one-half of its initial value, and afterwards more slowly (Fig. 10G). Generally, irregular variations appeared superimposed on the rapid part of the fall, but they were practically absent from the subsequent slow part. When test stimuli of lower frequency (10–15 shocks per sec.) were employed the tension rose slowly, exhibiting wavelets due to incomplete summation in the earlier part of the rise; after 2–4 sec. it reached a maximum and then declined smoothly (Fig. 10H). Some estimates of the activity of Preparation 3393 were made by finding the concentration needed to produce changes in the response to stimulation with 150 shocks per sec. similar to the changes produced by 1 μM prostigmine solution except for the differences just described. The figures are included in Table I.

Substance 37 had relatively little action on the responses to nerve stimulation except in concentrations which also reduced the response of muscle to direct stimulation. A 400–500 μM solution caused the form of the response to nerve stimulation with 100 or 150 shocks per sec. to

Fig. 10.

change in the same way that a 0·5–1·0 μM solution of prostigmine would have done, and, at the same time, it reduced the tension developed in the various parts of the response to one-half to two-thirds of what would have been expected had the preparation been treated instead with prostigmine.

(*a*)

The actions of eserine, Substance 38, and Preparation 1210/1 on the responses of "cold" frog preparations were similar to that of prostigmine, but no quantitative comparison was attempted.

(*b*)

In view of Kruta's [1935] findings that eserine was without action on the response of the frog's sciatic-gastrocnemius preparation to repetitive stimulation of the nerve by shocks at intervals of 0·4 sec., or at the rate of 30 per sec. for 2 sec. test periods, the actions of prostigmine and of eserine on the responses of that preparation were examined in a few experiments.

The preparation was dissected along with the lower half of the femur from a "warm" frog weighing about 20 g., and given a preliminary soaking in bicarbonate-buffered Ringer's solution. The apparatus and subsequent procedure were the same as in the experiments with sartorius preparations except for the following modifications. The partition *CC* and the non-polarizable electrode *G* were removed; the holder for the acetabulum was replaced by one suitable for the femur. Stimulation of the nerve was effected through the fluid electrodes used for the sartorius nerve. No attempt was made to stimulate the muscle directly because of the danger of stimulating nerve twigs at the same time.

The responses of Ringer-soaked preparations to stimulation with 50, 100, 150 shocks per sec. were very similar to those obtained from the Ringer-soaked sartorius preparations at the same frequencies. In order to minimize muscular fatigue, which would have complicated the responses, the test stimuli in each experiment were kept down to a minimum number and each of the shortest duration consistent with establishing the main changes that occurred. In no case were stimuli of duration longer than 15 sec. employed, nor were they applied to a preparation more often than once in 15 min. After the responses to two or three test stimuli had been recorded the preparation was immersed in a solution containing prostigmine in concentration up to 3 μM or eserine in concentration up to 10 μM, and, after an interval, the responses to further test stimuli recorded. The changes produced were similar to those produced in the responses of sartorius preparations, but the times which elapsed between the filling of the bath with the drug solution and the appearance of the changes were rather longer than with the sartorius preparations—presumably the extra time was required for the drugs to diffuse to the less accessible nerve endings in the isolated gastrocnemius [cf. Ing & Wright, 1931].

Fig. 10. 4. iii. 36. The action of Preparation 3393 upon the responses of a nerve-sartorius preparation. Time markings and signal as in Fig. 4. A, 5.9 p.m., nerve of Ringer-soaked preparation stimulated at frequency of 150 per sec. with just maximal shocks of time-constant 109 μsec. 5.40 p.m., bath drained and then filled with solution containing 6 μM Preparation 3393. B, 5.50 p.m., nerve stimulated as in A. C, 6.0 p.m., stimulation of nerve repeated. D, 6.10 p.m., stimulation of nerve repeated. E, 6.20 p.m., stimulation of nerve repeated. F, 6.30 p.m., stimulation of nerve repeated. G, 6.40 p.m., nerve stimulated as in A, but at frequency 24 per sec. H, 7.20 p.m., nerve stimulated as in A, but at frequency 14 per sec.

Comparison of the actions of phenyltrimethylammonium iodide and of curarine chloride on the responses of nerve-muscle preparations

During the earlier stages of the work on neuro-muscular transmission I thought that prostigmine, as a quaternary ammonium salt, might have a curare-like action in addition to its eserine-like action. As one way of obtaining an estimate of how far a curare-like action might be of importance I examined the action of phenyltrimethylammonium iodide, in concentrations between 0·1 and 10 μM, upon the responses of nerve stimulation, since the cation of that salt differs from the cation of prostigmine only in that the urethane grouping is absent. As reported in a preliminary communication [1936*a*] I found that no change in the responses occurred within half an hour of filling the bath with phenyltrimethylammonium iodide solution. However, after the discovery of the relatively great eserine-like activity of Preparation 1210/1, whose cation differs from that of phenyltrimethylammonium iodide in having an hydroxyl group in the meta position, it seemed possible that the action of phenyltrimethylammonium iodide in its lower effective concentrations might not be a purely curare-like one. [For references to the curare-like action of phenyltrimethylammonium salts see Trendelenberg, 1923; Ing & Wright, 1931.] I decided therefore, to examine further the action of this compound, carefully comparing it with that of curarine. The results are described below. The question that prompted the earlier experiments—whether prostigmine has a curare-like action will be discussed in the latter part of this paper.

When phenyltrimethylammonium iodide was used in concentration 10 μM the first clearly marked change in the response to nerve stimulation at a frequency of 150 per sec. occurred after about $\frac{3}{4}$ hr., when the tension developed in the secondary rise became reduced by 20–30 p.c. Often also the tension developed in the initial rise was reduced, but not by more than 10 p.c.: after attaining its maximum the tension began to fall earlier than in the responses of the untreated preparation (cf. Fig. 11A, B). During the next $\frac{1}{4}$ hr. the tension developed in the initial rise became reduced by about a further 5 p.c., a reduction not much greater than occurred in the responses of Ringer-soaked control preparations; on the other hand, the tension developed in the secondary rise became reduced by a further 10–20 p.c. In the course of another hour the responses of treated preparations suffered relatively no more change than did the responses of untreated preparations. When phenyltrimethylammonium iodide was used in a concentration of 50 μM the response

to nerve stimulation at a rate of 150 shocks per sec. was attacked, within 15–20 min. of filling the bath, to about the same extent that a 10 μM solution attacked it in 1½ hr. In about another 15 min. the maximum

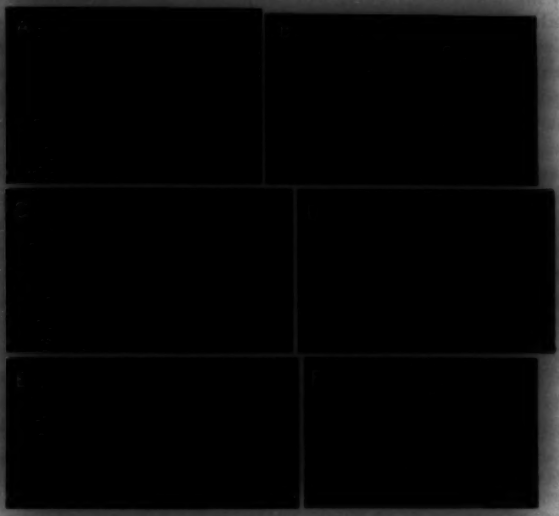


Fig. 11. 8. ii. 38. The action of phenyltrimethylammonium iodide upon the responses of a nerve-sartorius preparation. Time markings and signal as in Fig. 4. A, 2.45 p.m., nerve of Ringer-soaked preparation stimulated at a frequency of 150 per sec. with shocks of time-constant 73 μsec. 2.58 p.m., bath drained and then filled with solution containing 10 μM phenyltrimethylammonium iodide. B, 3.34 p.m., nerve stimulated as in A. 3.36 p.m., 0·05 c.c. of 5 mM phenyltrimethylammonium iodide solution added to the compartment which contained the greater part of the muscle to make the total concentration there 20 μM. C, 3.59 p.m., nerve stimulated as in A. 4.13 p.m., phenyltrimethylammonium iodide added to make the concentration in the compartment which contained the greater part of the muscle 50 μM. D, 4.38 p.m., nerve stimulated as in A. 5.51 p.m., phenyltrimethylammonium iodide concentration raised to 200 μM. E, 6.21 p.m., nerve stimulation as in A. 6.23 p.m., phenyltrimethylammonium iodide concentration raised to 300 μM. F, 7.10 p.m., nerve stimulated as in A.

tension attained in the initial rise did not suffer further reduction, but as soon as the maximum had been attained the tension began to fall rapidly, after a short time the fall slowed momentarily and afterwards continued at a constantly diminishing rate, the secondary rise having disappeared (Fig. 11D). Generally the tension fell to about 10 p.c. of its initial

maximum during 20 sec. of stimulation. In responses recorded about ½ hr. later the initial maximum tension had not become much more reduced, but the fall which followed the maximum had become faster and the momentary slowing had disappeared: the tension fell at a constantly diminishing rate and reached 5–10 p.c. of its initial value after 20 sec. of stimulation. The treatment of preparations with 200 μM phenyltrimethylammonium iodide solution for 10–15 min. affected the response to nerve stimulation with 150 shocks per sec. to the same extent that the 50 μM solution affected it in an hour. In the course of the next 2 hr. the maximum tension developed in the initial rise became reduced to two-thirds to one-third of that given by the untreated preparation, and the fall which followed almost immediately after the maximum became faster in successive responses.

The results of stimulation at lower frequencies do not require detailed description. When 50 or 100 shocks per sec. were used, phenyltrimethylammonium iodide in the smaller concentrations reduced the tension developed in the later part of the response but left that developed at the beginning of the response practically unchanged.

The respective actions of curarine chloride in concentrations 0·004–0·01, 0·04, 0·2–0·4 μM were indistinguishable from those of phenyltrimethylammonium iodide in concentrations 10, 50, and 200 μM.

Using a stimulation frequency of 150 per sec. a 50 μM solution of tetramethylammonium iodide was also found to attack the secondary rise in preference to the initial rise. Since, however, the spontaneous recovery of preparations partly paralysed with this substance in small concentrations [Ing & Wright, 1931] would have been an unnecessary complication no time was given to detailed experiments.

Antagonisms between eserine-like and curare-like substances

The action of curare-like substances on the responses of nerve-muscle preparations which had been treated with an eserine-like substance.

The method employed was either to add the curare-like substance dissolved in a small volume of fluid (0·01–0·1 c.c.) to the solution of eserine-like substance already in the compartment of the bath which contained the greater part of the sartorius muscle, or else to remove the solution from the bath, add the curare-like substance to the solution, and replace it in the bath. These methods were chosen in order to avoid upsetting any equilibrium which might have been established between the preparation and the solution of eserine-like compound bathing it. Preliminary tests showed that once the response of a preparation had attained a consistently reproducible form after filling the bath with

a solution of an eserine-like compound, subsequent withdrawal and replacement of the solution produced no further change in the response. The changes to be described were, therefore, due solely to the added curare-like substance.

The antagonisms between prostigmine and curarine will be described first. Changes in the form of the response were most marked when a stimulation frequency of 150 per sec. was employed, and when preparations which had been immersed in 0·3 μM prostigmine solution for about 2 hr. were treated with 1–2 μM curarine chloride solution. In responses recorded about 10 min. after addition of the curarine chloride the tension developed in the initial rise was restored to approximately that which had been obtained before treatment of the preparation with prostigmine. In some experiments, in which the preparation was sensitive to curarine, the tension fell rapidly soon after the initial maximum had been attained; in others, in which the preparation was less sensitive to curarine, the tension remained at its initial maximum for as much as a second before the rapid fall occurred. The tension developed in the subsequent secondary rise, in responses of either type, was reduced considerably below the augmented value that had resulted from the treatment with prostigmine (cf. Fig. 12A, B). In responses recorded 10–15 min. later the tension developed in the initial rise remained unchanged, but the rapid fall began almost immediately the maximum had been attained and continued until the tension had reached its pre-stimulation value. Often a momentary slowing occurred soon after the fall had begun. The secondary rise had disappeared (Fig. 12C). In responses recorded in the course of another $\frac{1}{2}$ hr. the tension attained in the initial rise suffered a little diminution, and the fall which followed the maximum became accelerated. When, however, curarine chloride was used in concentrations greater than about 4 μM the restoration of the initial tension was of a more temporary nature. The responses passed through the stages described above, and then, in subsequent ones, the rapid initial rise of tension became smaller and eventually disappeared.

In experiments with preparations which had been immersed for about 2 hr. in 3 or 30 μM prostigmine solution, curarine restored the initial tension developed in response to stimulation at a frequency of about 150 shocks per sec. in the same way as described above. I did not observe, however, in four experiments, any restoration of the secondary rise of tension after it had been suppressed by the action of prostigmine alone. The concentration of curarine chloride needed to restore the initial tension was the same, within the limits of the experimental method,

as that needed in the experiments with preparations which had been treated with 0·3 μM prostigmine solution, and, as in those experiments, the restoration produced by curarine chloride in concentrations greater than 4 μM was only a transient one. Rothberger [1901], and Langley & Kato [1915] have shown that the reversal of curare paralysis by eserine is also limited with respect to the amount of curare used.

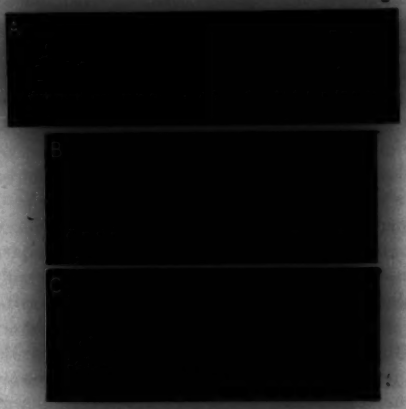


Fig. 12. 5. ii. 36. The action of curarine upon the responses of a nerve-sartorius preparation that had previously been treated with prostigmine. The time markings and signal as in Fig. 4. The preparation had been immersed in Ringer's solution containing 0·3 μM prostigmine for 1¼ hr. A, 4.52 p.m., nerve stimulated with maximal shocks of time-constant 109 μsec. at a frequency of 150 per sec. 4.55 p.m., 0·03 c.c. of millimolar curarine chloride solution added to the compartment of the bath which contained the greater part of the muscle to make the concentration there 1·2 μM. B, 5.2 p.m., nerve stimulated as in A. C, 5.14 p.m., nerve stimulated as in A.

Changes of the same type as those described in the two preceding paragraphs could also be produced in the responses of prostigmine-treated preparations by tetramethylammonium iodide in concentrations 100–200 μM, or phenyltrimethylammonium iodide in concentrations 250–500 μM. Similar changes in the response of a preparation which had been treated with any of the other eserine-like compounds were produced by any of the three curare-like compounds in the concentrations stated above. The antagonisms between the three curare-like compounds and Preparation 1210/1 were found not to differ qualitatively from the other antagonisms already mentioned. The restorative action of

phenyltrimethylammonium iodide upon the initial tension in the response of a preparation that had been treated with Preparation 1210/1 is shown in Fig. 9C, D.

The action of eserine-like substances upon the responses of preparations that had previously been treated with curare-like substances.

These experiments were made to determine whether the relative activities of the eserine-like substances in restoring the responses of preparations which had been nearly paralysed by a curare-like substance were of the same order as their activities on preparations which had merely been soaked in Ringer's solution. The method was the following one. The response of a Ringer-soaked preparation to maximal stimulation of its nerve for 1 sec. at a frequency of 100 per sec. was recorded two or three times at 10 min. intervals, then the bath was drained, filled with curarine chloride solution, and responses to further stimulation were recorded at 15 min. intervals. Soaking for about an hour in a 0·5–1·0 μM curarine chloride solution (usually 0·8 μM) sufficed to bring the preparation to a fairly steady or very slowly declining state in which the maximum tension developed in the response was reduced to about 10 p.c. of its pre-treatment value. When this state had been reached enough of the eserine-like substance whose action was to be examined, dissolved in a small volume of fluid, was added to the compartment of the bath which contained the greater part of the muscle to make the concentration there equal to the mean of the figures given in column 4 of Table I and then the recording of responses to further test periods of stimulation, at 15 min. intervals, was continued for about another hour. The results of these experiments are summarized in column 7 of Table I. The degree of recovery attained in each experiment is expressed as the ratio of the maximum tension developed by the preparation after treatment with an eserine-like compound to the tension developed by the preparation whilst under the influence of curarine alone, both tensions being stated as percentages of the maximum given by the preparation before it was treated with curarine chloride. It is probable that the depressant action of Substance 37 upon muscle partly accounts for the small degree of recovery produced in the experiments with this compound. A second series of experiments in which 300–400 μM phenyltrimethylammonium iodide was used instead of curarine chloride gave similar results. These are summarized in column 7 of Table I.

Discussion

The relation found in the experiments with isolated and Ringer-soaked nerve-muscle preparations between the frequency at which the nerve was stimulated and the form of the response is similar, in the main, to that found by Wedensky [1885, 1886, 1891] and by Hofmann [1903*a*] with frog's sciatic-gastrocnemius preparations. Wedensky used isolated preparations, and the majority of his experiments were complicated by muscular fatigue. Hofmann, on the other hand, in order to reduce muscular fatigue to a minimum, used preparations which enjoyed a blood supply, or isolated preparations which were allowed long rest intervals between stimuli. The variations in the form of the response exhibited by different preparations to stimulation at any one frequency, particularly frequencies higher than 60 per sec., were smaller in my experiments than in Hofmann's. There are three reasons which probably account for this difference: the relatively constant temperature at which the "warm" frogs were kept before use; the long preliminary soaking of the preparations in the Ringer's solution (it will be shown in a subsequent communication that changes in the composition of the Ringer's solution produce marked changes in the form of response); the maintenance of the bath at a fairly constant temperature during experiments. If a quantitative comparison is made of the amount of activity that Hofmann's circulated preparations and my isolated sartorius preparations could undergo before their reponses began to deteriorate then there appears a discrepancy which perhaps calls for comment. Hofmann states that his preparations began to show signs of "fatigue" after only 1–2 hr. of the activity resulting from test stimuli of 2–5 sec. duration applied at intervals of 5–10 min. Since Hofmann used intact unanæsthetized frogs it seems unlikely that the relatively early failure of his preparations can have been due to an inadequate blood supply; also he states that he took care not to impede the blood flow when fixing the leg under experiment. The most plausible explanation that I can suggest is based on some observations of Forbes & Ray [1923]. They found that nerve trunks which were allowed to remain in contact with injured tissues soon became inexcitable. Hofmann states that to prevent drying of the nerve in the intervals between the stimuli he lowered it into the wound. Possibly this procedure rendered some of the motor nerve fibres inexcitable and so gave the changes that he attributed to fatigue. Whether or not this is the correct explanation of the discrepancy, an isolated nerve-sartorius preparation, weighing about 70–80 mg. exclusive of bone,

which can develop and maintain a mean tension of about 50 g. for 15–20 stimulation periods each lasting about 15 sec., can hardly be regarded as so abnormal as not to merit investigation.

The question of whether prostigmine used in high concentrations causes the action current of nerve to increase must be left to further experiment. The observations that prostigmine in lower concentrations did not affect the nerve action current, that treatment of the nerve only of a nerve-muscle preparation produced no change in the response, and that the responses of nerve-muscle preparations which had been immersed in 3000 μM solution for 1½ hr. suffered no further change of form within another 14–16 hr., indicate that if prostigmine has any action it is very small. Evidence of a similar kind suggests that eserine and Substance 38 are without action on nerve. Also, none of the experiments with the remaining eserine-like compounds suggests that any of them affects nerve.

The changes produced by 0·2–0·4 μM curarine chloride in the responses of isolated nerve-sartorius preparations during the first 5 sec. of stimulation at various frequencies resembled those which Hofmann found to occur in the responses of his circulated sciatic-gastrocnemius preparations after the injection of small doses of curarine chloride [1903*a*]. More recently Bremer & Titeca [1935] have found that the muscles of preparations which had been curarized to a slightly greater degree than had Hofmann's were unable to maintain tension when their nerves were stimulated at a frequency of 10 per sec. Hofmann found also [1903*b*] that curarine chloride in greater doses brought preparations to a state in which a single nerve volley elicited no response from the muscle, but repetitive stimulation caused contraction. Boehm [1895] has examined the action of curarine on the responses of isolated sciatic-gastrocnemius preparations to stimulation at very low frequency—about one shock in 2 sec. He found that the maximum tension developed in successive twitches of a preparation which had been treated with a fairly heavy dose of curarine chloride quickly diminished to zero, whereas the tension developed in successive twitches of an untreated preparation diminished slowly. Boehm found that after allowing the curarine-treated preparation to rest for 10–15 min. the maximum tension recovered to its initial value and that it diminished more quickly in a second series of twitches. After a second rest interval of 10–15 min. the tension again recovered, and in a third series of twitches it diminished more quickly than in the second series.

There is no evidence that prostigmine exerts a true curare-like action at any of the concentrations examined [cf. Eccles, 1936]. By the use

of a stimulation frequency of about 150 per sec. it has been possible to show that prostigmine and curarine in their lower effective concentrations attack the responses of nerve-muscle preparations in different ways. When we come to consider the actions of the two drugs in relatively great concentrations, Boehm's observations on curarine-treated preparations may be contrasted with my observations on the tension developed in series of twitches given by preparations that had been treated with 3000 μM prostigmine solution. If a line be drawn through the summits of the twitches of a prostigmine-treated preparation the form of the tension curve so obtained is very similar to that exhibited by preparations which were treated with 0·3 μM prostigmine solution and afterwards stimulated at a frequency of 150 shocks per sec., although the time-scales differ considerably. So also, the form of curve passing through the summits of the twitches given by Boehm's curarine-treated preparations resembles that of the tension-time curves of preparations which were treated with curarine in very small concentration and afterwards stimulated at a frequency of 150 per sec. It seems probable that, by selection of a suitable stimulation frequency, characteristic differences between the responses of prostigmine-treated and curarine-treated preparations can always be demonstrated. Apart from the foregoing differences, the actions of eserine and of Substance 37, neither of which compounds contains a quaternary ammonium grouping, upon the form of response to nerve stimulation were qualitatively indistinguishable from that of prostigmine. Finally, 3000 μM prostigmine solution caused augmentation of the twitches elicited from a "cold" frog preparation.

The interpretation of the observations described in this paper in terms of the hypothesis that acetylcholine is the transmitter at motor nerve endings necessitates certain suppositions additional to those suggested in the opening paragraphs. Following the demonstration by Dale *et al.* that an ACh.-like substance is liberated at frog's motor endings, Brown [1937] has shown that the rapid intra-arterial injection of small amounts of ACh. into frog muscle sets up propagated excitation in the muscle: he has also shown that a temporary block of neuro-muscular transmission occurs after the injection of larger amounts of ACh. In Hofmann's experiments with circulated preparations and in mine with Ringer-soaked but otherwise untreated preparations, the relatively rapid fall of tension which followed the initial rise in the responses to nerve stimulation at a frequency of 100 per sec. and over may have been due to successive volleys liberating ACh. more quickly than the agencies responsible for its removal could deal with it. The secondary rise of

tension during the response to nerve stimulation at a frequency of about 150 per sec. might be attributed to a progressive diminution on the amount of ACh. liberated per nerve impulse, which would allow the rise and fall of ACh. concentration at the nerve ending to return to more normal limits. If an analogy is permitted then there is one which is very suggestive. Brown & Feldberg [1936] found that during stimulation of the pre-ganglionic fibres of the cat's superior cervical ganglion, which was perfused with Locke's solution containing eserine, the output of ACh. diminished rapidly and that within the same time the myogram of the nictitating membrane exhibited an initial rise, followed by a rapid fall, a slower secondary rise, and afterwards by a slow fall (cf. Fig. 5 above with Fig. 1 of their paper). To suppose that the ability of the frog's motor nerve ending to liberate ACh. in response to nerve impulses diminishes as a result of successive impulses and is restored again only after a relatively long rest interval would also be consistent with an observation of Wedensky's. He found [1886] that if the stimulation of a gastrocnemius-sciatic preparation at a frequency of about 130 per sec. were interrupted for a few seconds as soon as the secondary rise had become well developed and then recommenced, the maximum tension reached in the third rise was about the same as in the initial rise, but that after the third maximum the tension fell much more slowly than after the initial maximum and no secondary rise occurred. In my experiments with frog's nerve-muscle preparations the observation that the tension developed in response to nerve stimulation at low frequencies diminished more quickly than in response to direct stimulation of the muscle probably indicates that in the earlier volleys the amount of ACh. liberated per nerve impulse is supraliminal at most motor endings, but that later it becomes subliminal at some of the endings. If the action of curarine is to raise the threshold of the part of the muscle near the nerve ending to ACh. (and perhaps the threshold of other parts of the muscle also) the reduction of the later part of the response to nerve stimulation by curarine in low concentrations would be consistent with the liberation of supraliminal amounts of ACh. at most nerve endings by the earlier nerve impulses and with the liberation of smaller amounts of ACh. by later impulses. The action of prostigmine in causing the fall of tension which followed the initial rise in the response to nerve stimulation at a frequency of about 150 per sec. to occur earlier and to become deeper, also the augmentation of the secondary rise, can be explained either by the sensitization of the muscle to ACh. or by the inhibition of choline-esterase (see below). However, the observation that prostigmine caused

the secondary rise to occur earlier than in the responses of untreated preparations is the opposite of what would have been expected. To account for this it seems necessary to postulate that after a short time the part of the muscle fibre near the nerve ending becomes so adapted that the failure of the ACh. concentration to rise and fall within its normal limits no longer results in a junctional block. It seems, moreover, that such an adaptation can only occur in a limited degree, since increase of stimulation frequency or larger doses of prostigmine caused the secondary rise to disappear. The action of curarine in restoring the initial tension in the responses of preparations which had been treated with 0·3 μM prostigmine solution to stimulation at a frequency of about 150 per sec., and in reducing the secondary rise, are consistent with a raising of the threshold of muscle to ACh. and with the liberation of greater amounts of ACh. by the early nerve impulses than by the later ones.

The above interpretation would apply equally well to the actions of the remaining eserine-like and curare-like compounds.

Observations which differ little from those described here have been made by other workers using blood-circulated mammalian nerve-muscle preparations. The actions of eserine and of prostigmine in small and in moderate doses on the responses to nerve stimulation, for 3 sec. periods, at high and at low frequencies, have been examined by Briscoe [1936]. Rosenblueth *et al.* [1936], Bacq & Brown [1937], Rosenblueth & Morrison [1937] have also found that eserine or prostigmine in moderate doses causes the response to nerve stimulation at medium or high frequencies to become a twitchlike rise and fall, followed by nearly zero tension. Briscoe has shown that the initial part of such depressed responses may be restored by curarine (also Rosenblueth & Morrison). The action of curarine on the responses to stimulation of the nerves of preparations at various frequencies has been examined by Hofmann [1903*b*]. Brown *et al.* [1936] and Bacq & Brown [1937] have shown that eserine in small doses augments the twitches (see also below) in response to nerve stimulation at a rate of 6 shocks per min., and that larger doses cause augmentation of the first few twitches to be followed by depression. Rosenblueth *et al.* [1936] and Rosenblueth & Morrison [1937] have shown that if stimulation at low frequency is continued a slow recovery and a very slow fall follow the depression. Rosenblueth & Morrison have shown that if curare is used instead of eserine, then the heights of successive twitches diminish soon to zero.

Rosenblueth & Morrison, taking into account observations such as are summarized in the preceding paragraph, additional observations

described in their paper, the demonstration by Dale *et al.* that an ACh.-like substance is liberated at mammalian motor nerve endings, and the demonstration by Brown *et al.* [1936] that mammalian muscle may give twitchlike responses to rapidly injected ACh., have presented an interpretation of neuro-muscular transmission which differs in two respects from that given above of the observations on frog preparations. They have assumed that the action of eserine or prostigmine is to inhibit choline-esterase at the nerve endings. The results have not made it necessary to postulate that a partial adaptation can occur if the concentration of ACh. at the nerve ending fails to rise and fall within its normal limits. However, I think that the experimental evidence relative to this matter is not yet sufficiently clear. Rosenblueth & Morrison state that using cats anæsthetized with "Dial", stimulation of the nerve of a nerve-muscle preparation at a frequency of about 240 per sec. elicited a response in which a rapid fall, a secondary rise, and then a slow fall of tension followed the initial rise. Hofmann [1903*b*], on the other hand, found that rabbit nerve-muscle preparations (presumably he used unanæsthetized animals) gave steady responses to nerve stimulation at frequencies up to 260 per sec. Hofmann also found that when ether was used, there appeared in the response a rapid fall after the initial rise, and a secondary rise followed by a slower fall. From the evidence given in Hofmann's paper it is clear that this action of ether was not due to a local depression of the nerve trunk such as was studied by Wedensky [1903], but that it probably involves the whole nerve-muscle complex. As it seems unlikely that cat and rabbit preparations can differ much in their ability to respond to high-frequency stimulation it is desirable to know whether the type of response observed by Rosenblueth & Morrison is also characteristic of unanæsthetized cat preparations and, if it is, the effects of small doses of eserine-like substances on its form.

The "cold" frog preparations resembled cat preparations in that prostigmine augmented the early twitches in response to stimulation of the nerve at very low frequency [Brown *et al.* 1936; Rosenblueth & Morrison, 1937]. Evidence that the augmentation of the twitch is due to repetitive response of the muscle to a single nerve volley, and the conditions favouring this form of response by frog preparations, will be presented in a subsequent communication. The augmentation of the early part of the responses of "cold" frog preparations to nerve stimulation at low frequencies after treatment with prostigmine or eserine was probably due to multiple response to some of the nerve volleys. Rosenblueth & Morrison have examined the electrical responses of cat muscle

during low-frequency stimulation of its nerve (30 per sec.), and they found that prostigmine caused some of the responses to become multiple.

Marnay & Nachmansohn have obtained evidence that the choline-esterase present in frog muscle [1937*a*, *b*, *c*; 1938] and in lizard muscle [1937*d*, 1938] is concentrated near the nerve endings. Marnay *et al.* [1937], and Marnay & Nachmansohn [1938] have obtained similar evidence concerning the distribution of choline-esterase in mammalian muscle. Whilst in the experiments with frog sartorius preparations there was a fairly good parallel between the activities of the eserine-like compounds upon the responses to nerve stimulation at the rate of 150 shocks per sec. and in reversing curariform paralysis, there is not such a clear parallel between these estimates and Dr Stedman's estimates of the choline-esterase-inhibiting activities of the compounds. Prostigmine, Substance 38, and Preparation 3393, three substances which have anti-esterase activities of the same order, which contain the dimethylcarbamic ester grouping, and which are quaternary ammonium salts also exhibited approximately the same activity on neuro-muscular transmission. Substance 36 which possesses an anti-esterase activity one-third to one-half of that of prostigmine, which contains a phenylmethylcarbamic ester grouping, and which is a quaternary ammonium salt exhibited an activity about one-third of that of prostigmine on neuro-muscular transmission. Eserine, however, which has a rather higher anti-esterase activity than prostigmine, which contains a methylcarbamic ester grouping, and which is the salt of a tertiary base, exhibited only about one-fifth of the activity of prostigmine on neuro-muscular transmission. Also, Substance 37, which contains a dimethylcarbamic ester grouping, and which is the salt of a tertiary base exhibited on neuro-muscular transmission only about one-hundredth of the activity that would have been expected on the basis of its anti-esterase activity. Preparation 1210/1, which is both a phenol and a quaternary ammonium salt, exerted on neuro-muscular transmission an activity which was about 10,000 times what would have been expected from Dr Stedman's figures. Woldemar Hoffman [1886] found that phenol exerted an anti-curare action. Rothberger has shown [1902] that a number of phenols and cresols have anti-curare actions. Two main possibilities arise in connexion with Preparation 1210/1. It may be that this compound affects neuro-muscular transmission in some way other than by inhibition of choline-esterase; alternatively it may be that Dr Stedman's choline-esterase concentrates [Stedman & Stedman, 1935] contained substances which adsorbed Preparation 1210/1 and so protected the esterase. The relatively greater activity of the urethane

compounds which are also quaternary ammonium salts than of the urethane compounds which are also salts of tertiary bases, on neuro-muscular transmission may have been due to some specific action of the quaternary grouping, or to adsorption of the two kinds of compound to different extents by different parts of the nerve-muscle complex close to and remote from the nerve endings. It is to be noted that Bacq & Brown [1937] have examined the actions of a series of compounds of graded anti-esterase activity, using cat's blood-circulated nerve-muscle preparations, and that they found a fairly good parallel between anti-esterase activity and the minimal doses necessary to cause the response to a single nerve volley to become repetitive.

Summary

1. Treatment of frog's isolated nerve with prostigmine, eserine, or another eserine-like substance, produced practically no change in the action current elicited by maximal repetitive stimulation at frequencies ranging from 5 to 200 per sec. The action current was summed and observed by means of a galvanometer.

2. The myograms given by frog's nerve-sartorius preparations, when immersed in glucose-containing bicarbonate-buffered Ringer's solution, in response to maximal (nerve) stimulation, at high and at low frequencies, exhibited good reproducibilities, even after considerable activity. When stimulation frequencies between 15 and 200 per sec. were used three main types of response could be distinguished: with 15–60 per sec. the tension rose to a maximum and then fell slowly; with 100–180 per sec. the tension rose to a maximum, fell for a few seconds, rose again (secondary rise), and afterwards fell very slowly; with about 200 per sec. the tension rose to a maximum which was generally smaller than that attained with lower frequencies, and fell rapidly almost to zero. In the same experiments the responses of the sartorius muscle to direct stimulation were found to be influenced little by changes of stimulation frequency.

3. The actions of prostigmine, eserine, and five other eserine-like compounds, in various concentrations, upon the responses of nerve-sartorius preparations to stimulation at frequencies between 15 and 200 per sec. have been examined. The main action of any of these compounds was to lower the frequency required to produce the second and third types of response mentioned above in (2). In connexion with the hypothesis that acetylcholine is the chemical transmitter at motor nerve endings the action of small doses of the eserine-like substances on the

response to stimulation with 150 shocks per sec. seems of special interest: the tension developed in the initial part of the response was depressed, the minimum which followed was made smaller, and the secondary rise was augmented. The relative activities of prostigmine and the other eserine-like substances on the nerve-sartorius preparation do not exhibit a simple parallel to Dr Stedman's estimates of their inhibitory activities on his choline-esterase preparations.

4. The actions of curarine, and of phenyltrimethylammonium iodide, in low concentrations, upon the responses of nerve-sartorius preparations, to stimulation at frequencies between 50 and 150 per sec. have been examined. These substances, in contrast to the eserine-like substances, little affected the initial part of the response to stimulation at a rate of 150 per sec., but they reduced the secondary rise.

5. The actions of curarine, of phenyltrimethylammonium iodide, and of tetramethylammonium iodide, upon the responses of nerve-sartorius preparations which had previously been treated with an eserine-like compound, to nerve stimulation have been described.

6. The relative activities of the eserine-like compounds in reversing paralysis due to curarine or to phenyltrimethylammonium iodide were of the same order as their activities on the responses of Ringer-soaked preparations (see (3) above).

My sincere thanks are due to Mr E. B. Verney for the facilities provided in his laboratory, where approximately three-quarters of this work was done; to Prof. J. H. Gaddum for similar facilities in London, and for reading through a draft of the greater part of this paper and making several suggestions for its improvement.

I am indebted to Roche Products Ltd., London, and Hoffmann-La Roche, and Co. Ltd., Basle, for supplies of the eserine-like substances, and particularly for synthesizing Preparation 3393. I am also indebted to the Government Grants Committees of the Royal Society and to the Research Grants Committee of the Senate of the University of London (Thomas Smythe Hughes and Beaverbrook Funds) for allowing me to retain apparatus which had been purchased with grants from them and used for other earlier work. Finally, I am indebted to the Librarians at University College and elsewhere for making copies of Wedensky's monograph and Woldemar Hoffman's thesis available to me.

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J. Physiol. (1938) 93, 263–275 612.284

THE DEPENDENCE OF THE ACTIVITY OF THE "APNEUSTIC CENTRE" ON THE CARBON DIOXIDE OF THE ARTERIAL BLOOD

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(*Received* 9 *May* 1938)

In this paper are described experiments intended to throw further light on the nature of the central nervous mechanisms which bring about the apneuses, i.e. those characteristic and profound alterations of the respiratory activity, the "inspiratory cramps", which were first described by Markwald [1887, 1890] after division of the vagi in animals decerebrated at the level of the pons. Henderson & Sweet [1929] and Hess [1931] have recently suggested that the phenomenon is a manifestation of decerebrate rigidity: the transection of the pons, according to these authors, simply abolishes the well-known inhibitory influence of the red nucleus upon the tonus-producing centres of decerebrate rigidity in the medulla, and the reason why regular respiration still goes on, only to cease and give way to apneuses, when the vagi are cut as well, is that the afferent pulmonary vagus exercises a specific inhibitory influence upon those centres of decerebrate rigidity which control the muscles of inspiration.

It was shown, in a previous paper [Stella, 1938], that this view finds no support in experiment since (*a*) the centre in the brain stem whose removal, after division of the vagi, caused the cessation of regular respiration and the occurrence of the apneuses was not the red nucleus: a complete transverse section of the mid-brain immediately caudal to this nucleus, in the vagotomized cat, did not give rise to any sign of apneusis, which, on the contrary, invariably developed after sections in the pons, at least 2–3 mm. below its upper border; (*b*) the development of apneusis did not depend, as does decerebrate rigidity, on afferent influences from the periphery, i.e. on proprioceptive impulses from the respiratory muscles or from the labyrinths. It was still observed in cats in which the last four cranial nerves and all dorsal roots of the cervical and thoracic

cord had been previously severed, as well as in animals in which the V, VII and VIII cranial nerves had been divided together with all dorsal roots of the thoracic cord.

In the present state of our knowledge the most probable explanation of apneusis still remains that put forward by Markwald [1887, 1890], and accepted among others by Lewandowsky [1896], Asher & Lüscher [1899] and Lumsden [1922, 1923]. According to these authors apneusis is nothing else but the expression of the natural tendency of the inspiratory centre (Lumsden's "apneustic centre") to persist in an active tonic state when, under the experimental conditions, it has become released from both the reflex inhibitory influence of the pulmonary vagus, and from the automatic inhibitory influence of a higher centre (Lumsden's "pneumotaxic centre") either in the posterior colliculi [Markwald, Lewandowsky and others) or in the upper region of the pons [Lumsden, 1922; Stella, 1938]. These two influences, in the intact animal, rhythmically inhibit the apneustic centre and thus bring about normal respiration.

Henderson & Sweet [1929], Teregulow [1929] and Hess [1931] objected to this theory on the ground that, while the apneustic centre in the brain stem lies, according to Lumsden, in the region of the *striæ acousticæ*, sections of the medulla below this level in cats with intact vagi did not modify quiet natural breathing, thus suggesting that the apneustic centre is not responsible for normal respiration. Under similar experimental conditions Lumsden had observed not a normal type of breathing as was described by the authors mentioned above, but a series of "gasps", i.e. of respiratory acts of a character very different from the normal which he attributed to a sort of primitive centre, different from that on which normal respiration depends, and probably playing, under normal conditions, no part at all or only a subordinate one (the "gasping centre"). The writer under similar experimental conditions also observed only gasps, never normal respiratory acts [1937–8]. In any case, the persistence of normal respiration after decerebration below the level of the *striæ acousticæ*, even if confirmed, would not necessarily contradict Markwald's theory of respiration but might simply indicate that the apneustic centre reaches down in the medulla to a level lower than had been suggested by Lumsden, and it may be suspected that, if Henderson & Sweet and Teregulow had cut the vagi in their animals, decerebrated below the *striæ acousticæ*, natural quiet breathing might have given place to apneuses, owing to the preservation of the apneustic centre, or of part of it.

It may be inferred from these considerations that serial sections of the medulla do not afford the best method of solving the already very old question, as to whether or not the apneustic centre represents the centre of inspiration. It seemed that more information on this point might be derived from a study of the response of the apneustic centre to changes in the gaseous composition of the blood.

To my knowledge this question has never been submitted to a systematic investigation, and the few observations scattered in the literature are not sufficient to furnish the desired information. Markwald [1887] and Lumsden [1923] noticed, as was to be expected, that when the arterial blood owing to the standstill of respiration becomes very poor in oxygen the apneustic centre fails, but recovers again if oxygen is again supplied. On the other hand, if asphyxia is prevented by continuous insufflation of the lung with air or a mixture containing oxygen, each apneusis can be made to last for 30 min. or more, thus suggesting a great degree of indefatigability of the centres involved. Little is known of the effect of variations of the CO_2 tension of the arterial blood: Lumsden using a mixture containing 14 p.c. CO_2 and 28 p.c. O_2 obtained a very small increase in depth of the apneuses (about 25 p.c.), but Henderson & Sweet using a mixture of 7 p.c. CO_2 in air did not notice any change in the depth of the apneuses and only some shortening in their duration. Henderson & Sweet appear, moreover, to have tried the effect of diminishing the CO_2 tension of the blood below the usual normal values and to have obtained only negative results: in the protocols of their experiments, they once noted "apneusis persisting in spite of prolonged artificial respiration", and, in another case, "apneustic respiration even with pump operating".

Method

This research has been carried out upon cats, and has consisted in observing whether the development and depth (i.e. the degree of expansion of the chest) of the apneusis which ensues on blocking the vagi in an animal decerebrated below the "pneumotaxic centre" varies in accordance with various degrees of respiratory activity at the moment of vagus blocking. It has been noted previously [Stella, 1937] that so long as the vagi are functional, section in the pons below the "pneumotaxic centre" does not essentially modify the respiratory responses to changes of CO_2 tension of the arterial blood. Now under these conditions, the only inhibitory influence which, according to the view of Markwald and others, is responsible for transforming apneusis into rhythmical respiration, is that of the afferent pulmonary vagus. But it has been

shown by Adrian [1933] that the degree of activity of this nerve from stretch receptors in the lungs is directly proportional to the volume of the lungs, and is not affected by very large changes in the CO_2 or O_2 tension of the blood. Therefore, if the apneustic centre is actually the inspiratory centre, the large variations in the depth of breathing caused by changes in the gaseous composition of the blood, and indeed the apnœa from acapnia, must be due either to correspondingly large variations of the degree of activity of the apneustic centre under the different gaseous conditions, or else if the apneustic activity is itself independent of the gaseous composition of the blood to the existence of an inverse relationship between the tension of CO_2, on the one hand, and the susceptibility of the apneustic centre to the inhibitory discharges of the pulmonary vagus on the other.

The cats were decerebrated by the trephine method, under ether anæsthesia, by transverse section of the pons, 2–3 mm. below its upper border, ventrally, and immediately above the emergence of the cerebellar peduncles, dorsally. During the operation the common carotids were kept clamped, and the vertebrals slightly compressed. The tentorium cerebelli was usually removed, and in many cases the cerebellum was split along the mid-line, or was extirpated.

To block the vagi, cold thermodes were used, at 3–4° C.: for this purpose the vagi were exposed in the neck and freed for a length of about 3–4 cm., and loose loops passed round them, so that they could be easily lifted and placed on the thermodes whenever desired. The blocking of the vagi was carried out at intervals for only short periods at a time (15–120 sec.) repeated during the different conditions of (*a*) quiet breathing, (*b*) respiratory stimulation due to increased CO_2 in the inhaled air or "rebreathing" through a long rubber tube, or (*c*) complete apnœa induced by previous artificial over-ventilation of the lungs. This method of temporary blocking of the vagi had the great advantage over that of section of the nerves, as carried out by previous authors, in that it allowed an immediate direct comparison between the effect of CO_2 changes on the respiratory activity on the one hand, and on apneusis on the other.

The respiratory movements were recorded by means of Marey's tambours placed on the lateral sides of the thorax, or on the xyphoid. Blood-pressure records were taken from the femoral artery, by means of a Hg manometer. Throughout each observation on the behaviour of apneusis, observations were made at the same time on the state of decerebrate rigidity of the limbs, which was usually well developed.

Results

Effect upon apneusis of increasing the CO_2 *content of the inhaled air*

In all those animals, in which respiration, after transection of the pons, retained a normal degree of sensitivity towards change of the CO_2 content of the inspired air, the apneuses which ensued on cooling the vagi varied greatly in depth according to whether respiration was, at the time, quiet or stimulated. In the latter case the apneuses were deeper, and the more so, within limits, the greater the increase of the respiratory depth at the moment of cooling. Indeed, except for the extreme degrees

Fig. 1. Respiratory freeing of a 2·5 kg. female cat, decerebrated along a plane passing through the posterior part of the posterior colliculi and about 2 mm. below the upper border of the pons. Mean blood pressure = 130 mm. Hg. Between *c* and *w*: blocking of the vagi in the neck, by cold thermodes. Between the arrows the animal was made to inhale from a Douglas bag a mixture of air and 8 p.c. CO_2. Time: 4 sec. In this and all subsequent records—read from left to right: inspiration upward; expiration downward.

of respiratory stimulation, the relative increase of the depth of the apneuses was the same as that of the respiratory movements. On the other hand, so long as respiration was not interfered with and remained constant in rate and depth, the apneuses, tested several times in the course of hours, were always found to remain of the same constant depth.

As seen in Fig. 1, which represents a typical experiment, the increase of the depth of apneuses brought about by CO_2 excess in the air can be very considerable indeed, the average maximum observed in the course of this research having been of the order of three to four times the level during quiet breathing. In Fig. 1 between (*a*) and (*b*) the animal was made to inhale, for a short period, air and 10 p.c. CO_2 from a Douglas bag.

It will further be seen in the figure that with the washing out of the CO_2 excess from the system, by the animal's enhanced pulmonary ventilation, the gradual subsidence of the respiratory stimulation was accompanied by a corresponding gradual return of the apneuses to the initial level (*c*), (*d*) and (*e*). On the other hand, when the CO_2 content of the inhaled air was too small to cause stimulation of respiration, it was also without any apparent effect upon the apneuses. The range of CO_2 concentrations which were sufficient to bring about the maximal increase of apneustic depth were without any constant influence upon the degree of decerebrate rigidity of the limbs. Taking the depth of apneusis as a measure of the degree of activity of the apneustic centre, the results indicate that this centre is as specifically sensitive to increase of CO_2 tension of the arterial blood, as is the central respiratory mechanism as a whole.

Effect of "rebreathing"

The same quantitative relation as above noted, between the increase in the depth of apneuses and that of the respiratory movements in response to increased CO_2 in the air, was also observed when respiration

Fig. 2. 2 kg. male cat: section in upper region of pons. Between (*c*) and (*w*): cooling of the vagi. *A*, during quiet respiration in air; B, during "rebreathing" through a tracheal tube of 7 mm. bore, and 50 cm. length. Time: 1 sec.

was stimulated by "rebreathing" (breathing through a long tracheal cannula, 7 mm. bore, 50 cm. length) (Fig. 2). Since in this case the effect was partly due to oxygen lack, the results suggest that the apneustic centre, like the respiratory centres, may also be influenced reflexly, from the chemical receptors of the carotid sinus. Control experiments were carried out in which the vagi were blocked by a 1 p.c. solution of novocaine instead of by cooling, so as to remove the possibility of nerve irritation by the latter procedure. The solution was applied to a tract of the nerves deprived of their sheaths so as to make the penetration and removal of the novocaine quicker. The results confirmed those described above.

Artificial over-ventilation of the lungs

The great importance of the respiratory conditions of the blood as a factor which affects the degree of activity of the apneustic centre is further shown by the observations described below, on the effect upon apneusis of a drop in the CO_2 tension of the arterial blood below the normal levels, as brought about by excessive artificial ventilation of the lungs. When the vagi were blocked during the transitory period of apnœa thus induced, there was at first no sign of apneusis (Fig. 3). This only began to develop, very slowly and gradually, after some time,

Fig. 3. 3 kg. male cat: section in upper region of pons. Shows effect of blocking the vagi (at the arrow) (*A*) during quiet breathing; and (*B*) during the state of apnœa induced by artificial over-ventilation (*E*). Time: 1 sec.

depending on the magnitude of the previous ventilation. The delay in the appearance of apneusis was, moreover, about the same as the delay in the return of spontaneous respiration after an equal amount of pulmonary ventilation when the vagi were not cooled. Indeed the period of apnœa was as a rule slightly longer (a few seconds) than the delay in the rising of apneusis, as is well seen in the typical experiment of Fig. 4 by comparing (*B*) with (*D*) and (*C*) with (*E*). It will also be noticed that the slow rate of development of apneusis clearly corresponds to the slow gradual return of the respiratory activity to its original degree.

To make sure that this transitory disappearance of the apneustic activity was not due to a summation effect of the inhibitory discharges from the pulmonary vagi, set forth by the repeated forcible insufflation

of the lungs, control experiments were performed in which these nerves were placed on the cold thermodes at the very beginning of the artificial respiration. The results thus obtained were in all respects the same as those described above. The delay in the appearance of apneusis was, moreover, for any given amount of ventilation entirely independent of whether the vagi had been blocked from the beginning, or only after the ventilation was discontinued. The effect, therefore, had nothing to do with

Fig. 4. 2·5 kg. female cat: section in upper region of pons. Between (c) and (w) = blocking of the vagi in the neck, by cold thermodes. *V* = artificial over-ventilation. Time: 4 sec.

afferent inhibitory influences from the vagi. Also section of the phrenics in the neck to abolish the afferent impulses from the diaphragm did not modify the results.

That afferent impulses set up in other parts of the respiratory system and reaching the centres through other nerve channels were not mainly responsible for the subsidence of the apneustic activity is further shown by over-ventilating the lungs with air containing a high percentage of CO_2, when the apneustic activity, far from being reduced or abolished, was, on the contrary, greatly enhanced. A typical experiment is given in Fig. 5 in which the vagi were kept blocked while artificial ventilation was

carried out alternately with air, or with a mixture of 6 p.c. CO_2 and air from a Douglas bag. It will be seen that whereas the ventilation with air caused the chest to assume a resting expiratory position, thus indicating the absence of apneustic activity, the mixture of CO_2 and air, on the contrary, rapidly caused the chest to expand and remain in a forcible inspiratory position, in fact, in an apneustic position deeper than that obtained following quiet breathing. The holding in abeyance of the

Fig. 5. Same animal of Fig. 3. Between (*c*) and (*w*): blocking of the vagi by cold thermodes. In (*B*) until (*X*): artificial over-ventilation of the lungs by pump. At (*M*) and (*M'*) a mixture of 6 p.c. CO_2 and air is substituted for air. At (*A*) air is substituted for the mixture. Time: 4 sec.

apneustic activity by over-ventilation with air, and its revival by air containing CO_2, could be repeated indefinitely. The effect is clearly not mechanical but primarily chemical in origin and due respectively to excessive removal or excessive accumulation of CO_2.

The minimum amount of over-ventilation with air which was necessary to produce the transitory disappearance of "apneustic" activity was comparatively very small, indeed very far from such as might have induced a general acapnial shock [Rosenthal, 1882; Henderson, 1908; Dale & Evans, 1922]. It caused a drop of the arterial pressure of no more than 10–15 mm. Hg, and did not interfere at all with the corneal and nose reflexes nor with the degree of decerebrate rigidity of the elbow and knee joints. Indeed these functions were unaffected by much larger

ventilations, such as would be followed by a delay of 40–50 sec. in the return of apneustic activity.

In seven experiments out of a total of forty-four neither increase of the CO_2 content of the air, nor protracted artificial over-ventilation, had much influence upon the extent of the respiratory movements. This condition of apparent insensitivity of the respiratory centres to CO_2

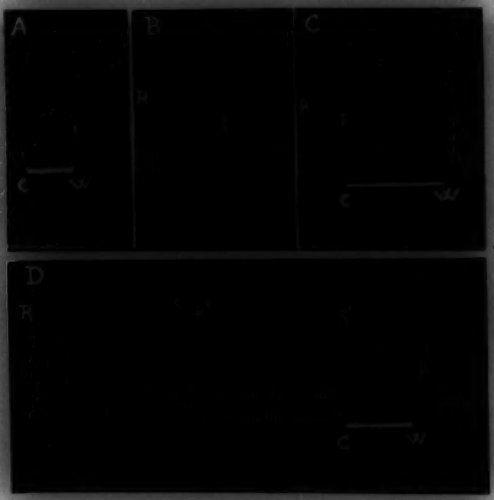

Fig. 6. 2·3 kg. male cat: section in upper region of pons. Between (*c*) and (*w*): blocking of the vagi by cold thermodes. *R* = artificial pulmonary over-ventilation discontinued at (*S*) and at (*S'*). Mean blood pressure = 140 mm. Hg. Time: 1 sec. In *D*, taken 2 hours after *A*, *B*, *C*; it is seen that over-ventilation fails both in causing apnœa (*S*–*R'*) and in delaying the development of apneusis (*c*–*w*).

change was, in some instances, noticed immediately after sectioning the brain stem; in other instances it developed gradually in the course of hours. In the latter case it could be seen that quiet respiration in air was also becoming gradually deeper and more frequent. The level of the section had no part in the production of the condition. It was once observed after a high section, above the pons, which had left the "pneumotaxic centre" intact and functional; while, on the other hand, section in the inferior region of the pons, i.e. much lower than those made in this

research, would, as a rule, leave the respiratory sensitivity to CO_2 almost unimpaired [Stella, 1937]. This exceptional behaviour of respiration was therefore most probably due to local circulatory derangement and consequently to a continuous state of stimulation of the centres from local anæmia or asphyxia. Whatever its cause, however, it is important to note that it was invariably associated with a similar degree of insensitivity to CO_2 change, on the part of the "apneustic centre", as is shown in Fig. 6. In this, pulmonary ventilation of an amplitude and rate which in (*B*) had been sufficient to cause transitory diminution of the respiratory activity, when repeated 2 hours later (*D*) failed to do so in spite of the fact that here it was continued for a much longer period. The apneustic activity, like the respiratory movements, was also not reduced as a result of the over-ventilation, as is seen by comparing the apneusis in (*C*) taken soon after (*B*) with that in (*D*).

On all the results described in this paper, the condition of the cerebellum (i.e. whether it was left intact, or split, at the mid-line, or extirpated) seemed immaterial.

Discussion

Although in this research no direct measurements of the blood CO_2 were carried out, it is safe to assume that the variations in the activity of the apneustic centre observed here in response to over-ventilation, or to increase of the CO_2 content of the inhaled air, were due, like the parallel variations of the respiratory activity, to changes in the tension of that gas in the arterial blood [Haldane & Priestley, 1905].

The apneustic centre thus differs also in this respect from the centres of decerebrate rigidity to which Henderson & Sweet [1929] and Hess [1931] attribute the phenomenon of apneusis.

In the light of the present findings, it is most probable that the failure of previous investigators [Lumsden, 1922, 1923; Henderson & Sweet, 1929] to recognize the great importance of the CO_2 tension as the main factor which determines the degree of activity of the apneustic centre, was due to the fact that their decerebrate preparations happened to be in that peculiar condition, occasionally observed in the course of this research, and which seems to be brought about by local circulatory derangement from the operation in which also regular respiration becomes, to a large extent, independent of the CO_2 tension of the arterial blood; a condition which these authors could not recognize, since in their experiments the vagi were cut, and no regular respiration, therefore, was possible.

It is, therefore, no longer possible to visualize, with Henderson & Sweet and Hess, apneusis as being the expression of an activity different from the respiratory one. On the other hand, the constant parallelism which was shown to exist at any moment between the degree of the apneustic activity and that of the respiratory activity; the fact that the depth of apneusis is always at least as great, but usually greater than that of the respiratory movements; the fact that after a period of over-ventilation, the apneustic activity returns a little earlier than regular respiration; all these observations fit very well with the old view of Markwald [1890], who regarded apneusis as the expression of the unrestrained activity of the inspiratory centre, and regular respiration as a rhythmically interrupted apneusis.

The results also show that the afferent discharges of the pulmonary vagus besides rhythmically interrupting the fundamental apneustic activity, moreover maintain it under a continuous state of restraint, which diminishes only under the conditions of extreme respiratory stimulation.

All this applies to the respiration which is observed after exclusion of the pneumotaxic centre; but it is unlikely that the phenomena would be essentially different when this centre is also functional. If the marked deepening and slowing of respiration after division of the vagi in an intact animal be compared with the persistence of a normal rate and depth of respiration, when, with vagi intact, the pneumotaxic centre is cut off, it will appear that the inhibitory influence of the latter is not so powerful as that of the vagi, and that the pneumotaxic centre, therefore, cannot have the paramount importance in the regulation of respiration attributed to it by Lumsden [1922–3].

Summary

1. The degree of activity of the "apneustic centre", as judged from the rate of development and depth of the apneusis which ensues on blocking the vagi in an animal decerebrated below the "pneumotaxic centre", is influenced as greatly, and in the same direction by very small change of the CO_2 tension of the blood as is the respiratory activity itself: thus, during apnœa from acapnia the apneustic activity is absent or extremely small; while during the intense respiratory stimulation induced by breathing air containing 8–10 p.c. CO_2, the apneustic activity reaches to a degree about 3–4 times as high as that during quiet respiration. These marked changes of the apneustic activity occur within such ranges of CO_2 tension in the blood as do not appreciably affect the reflex

activities of the central nervous system, or the degree of decerebrate rigidity of the limbs.

2. From these and other observations described in this paper, it is concluded that the apneustic centre is the inspiratory centre, and that normally its activity is not only rhythmically interrupted by the afferent inhibitory discharges of the pulmonary vagus, but also kept by these nerves under a continuous state of partial restraint.

The expenses of this research were defrayed out of a grant from the Dixon Fund of the University of London, for which the author is much indebted.

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A MICRO BLOOD VOLUME METHOD USING A BLUE DYE AND PHOTOCELL

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(*Received* 24 *May* 1938)

The method described is designed to eliminate as far as possible the factors causing inaccuracy in blood volume determinations while retaining maximum simplicity. The advantages of the method are:

(1) that the errors caused by hæmoglobin and serum pigment are automatically eliminated almost completely;

(2) that the amount of blood required for the samples is very small, making it suitable for small animals;

(3) that it uses a blue dye peculiarly suitable for plasma volume determination;

(4) that the measurement of colour density is made by means of a simple photocell colorimeter, thus eliminating the ordinary visual colorimeter which may be liable to serious error [Gregersen, 1938].[1]

In the measurement of the circulating blood volume the validity of any dye injection method depends upon:

(1) the injection of a suitable dye,

(2) complete mixing of the dye with the blood,

(3) accurate measurement of the dilution of the dye in the plasma,

(4) accurate measurement of the ratio of blood cells to plasma or hæmocrit value.

The criteria of a suitable dye are that it must (1) be non-toxic, (2) not be absorbed by the cellular elements of the blood, (3) have a slow rate of

[1] During the preparation of this paper Gibson & Evelyn [1938] published a photocell method for blood volume determination, derived from the spectrophotometric procedure of Gibson & Evans [1937] which has many points in common with that described here. We put our experience on record for two reasons: first, that this method provides an independent confirmation of the underlying principles common to both; second, that the differences in the two methods are not negligible. This method has important advantages in the colour filter used, the lack of need for a separate correction for hæmolysis, the volume of serum required, and the knowledge of an overall control covering all the sources of error.

disappearance from the blood stream, and (4) have physical characteristics suitable for accurate measurement.

In order to obtain a sample of blood completely mixed with the dye two quantities must be known; the time required for the injected dye to become homogeneously distributed and the rate of disappearance of the dye.

Accurate colorimetric measurement of the dilution of dye in a sample of plasma is made difficult by the presence of (1) plasma colour, (2) hæmoglobin, (3) lipæmia, (4) residual dye, and (5) preparation of the standard for colour comparison [Gregersen *et al.* 1935; Gregersen, 1938].

Accuracy of the hæmatocrit measurement depends upon complete packing of the cells and use of an anticoagulant which allows correct estimation of the normal proportion of cells to plasma.

Fat particles in the plasma will cause an error in any dye injection method for measuring plasma volume.

The results reported in this paper were obtained using sheep blood but should be equally applicable to other bloods, provided the same controls are observed.

Choice and suitability of dye

Dye. The dye used is T 1824 or Evans Blue, the use of which was suggested by H. M. Evans in 1920, extensively studied for plasma volume work by Gregersen and co-workers, and introduced into clinical work by Gibson & Evans [1937].

Toxicity. The dye is non-toxic in dosage up to 20 mg./kg. [Gibson & Gregersen, 1935]. It has been given to several hundred human subjects in dosage of 0·2 mg./kg. without untoward effect [Gibson & Evans, 1937; Thompson *et al.* 1938], and to sheep in dosage of 0·5–1·0 mg./kg. without effect on weight curve [Barcroft & Kennedy, 1938].

Disappearance rate. The average disappearance rate in both dog and man is 5–8 p.c. per hr. [Gregersen *et al.* 1935]. Comparison of a large series of vital dyes used for plasma-volume determination has shown that as a group the red vital dyes disappear from the blood stream 65 p.c. more rapidly during the first hour than T 1824 [Dawson *et al.* 1920].

Stability of dye. Aqueous filtered solutions of T 1824 are remarkably stable and retain a constant colour value for several months [Gibson & Evans, 1937]. This allows use of the same stock solution over a long period. Most of the red vital dyes fade within a few hours.

Standardizing dye solution. It is essential that both calibrating sample (or standard) and test sample be made up in solvents in which the dye has the same light absorption for a given dilution [Gregersen & Gibson, 1937]. This condition, which has not been fulfilled in many previous dye methods, can be satisfied by the choice of suitable concentrations of salt and plasma. Solutions of T 1824 made up in 0·9 p.c. NaCl containing 20 p.c. plasma has been found to give the same light absorption as an equal concentration of dye in whole plasma.

T 1824	In 0·9 p.c. NaCl containing 20 p.c. plasma	In whole plasma
Stock solution A	35·6	35·4
Stock solution B	36·4	36·4

Light absorption of the dye is independent of individual variations in the sheep plasma with which it is diluted as shown in Table I.

TABLE I. Light absorption of T 1824; stock dye solution B diluted 250 times in 0·9 p.c. NaCl containing 20 p.c. plasma

Date	Sheep no.	Colour reading
7 March 1938	309	36·4
9 March 1938	337	36·5
11 March 1938	285	36·4
17 March 1938	262	36·6
23 March 1938	329	36·5
23 March 1938	289	35·5
28 March 1938	298	36·4
29 March 1938	354	36·6
30 March 1938	325	36·7

Greatest variation is 0·3 unit which is equivalent to 1 p.c. error in plasma volume. The reproducibility of the calibration readings eliminates the necessity of frequent recalibration of the stock dye solution.

Effect of blood cells and clotting on dye. Absorption of the dye by the cellular elements of the blood is negligible, and coagulation of blood does not effect the concentration of the dye in the plasma [Gregersen & Schiro, 1938]. This allows the use of clotted blood samples.

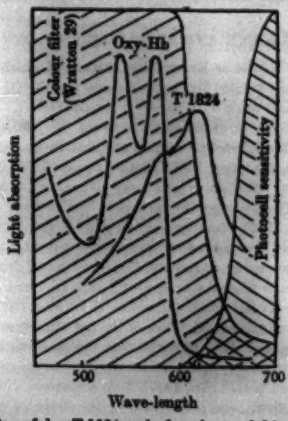

Fig. 1. Absorption spectra of dye T 1824 and of oxyhæmoglobin. The shaded area on the left represents light absorption of Wratten Filter No. 29; that on the right the decrease in sensitivity of the photocell.

Colour filter. As seen in Fig. 1, the combination of the colour filter (Wratten 29 F) and the photocell sensitivity results in the transmission of effective light only from about 610 to 690 mμ. wave-length. Since both

oxyhæmoglobin and serum pigment absorb very little light in this region their presence causes a negligible error in the colour readings. Thus, the effective light absorption is due almost entirely to the dye which has its absorption peak at 620 mμ. It has been found empirically that the dye follows Beer's law under the conditions of the experiment; that is, the negative logarithm of the transmitted light is proportional to the concentration of the dye.[1] This is shown in Fig. 2.

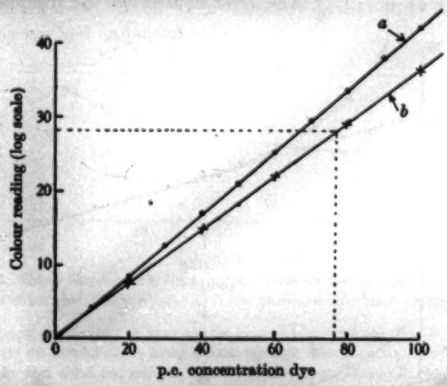

Fig. 2. The validity of Beer's law for T 1824 under the experimental conditions employed, and the equivalence of 20 p.c. plasma and whole plasma as dye solvents. Curve *a*: dye made up in 0·9 p.c. saline; "100 p.c." dye concentration = 40 mg./litre. Curve *b*: dye made up in two different strengths of plasma. × = 20 p.c. plasma in 0·9 p.c. saline. ● = whole plasma.

Mixing time. Large errors may be obtained if the dyed sample is withdrawn before the injected dye is thoroughly mixed with the circulating blood. Since the length of time required for complete mixing varies in different species of animals and even in the same animal with changes in the circulation, it is important to determine this interval accurately. Fig. 3 shows the obvious fallacy of obtaining the correct dilution before mixing is complete.

Disappearance rate curve and extrapolation. Immediately after the injection of any dye it begins to disappear from the circulating blood.

[1] Beer's law theoretically holds only for monochromatic light or over a wave-length region where the absorption coefficient is sensibly constant. The combination of sharp cut off of photocell sensitivity on one side and of light transmission by the red filter on the other, approximates this condition, as shown by the curves (Fig. 2).

This takes place during the process of mixing and continues thereafter at a uniform rate. The error caused by the small amount of dye which has disappeared during the interval up to the time of withdrawal of samples may be corrected by extrapolation back along the line represented by the disappearance rate curve to zero time or time of dye injection (Fig. 3). This theoretical value represents the dilution of dye if it were completely mixed at the moment of injection [Sunderman & Austin, 1936; Gibson & Evans, 1937]. The error caused by the disappearance of

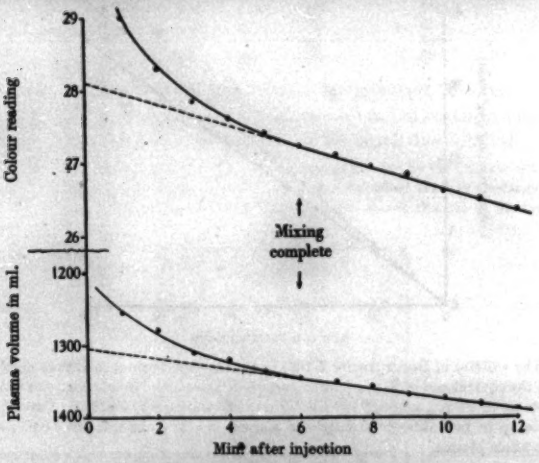

Fig. 3. Mixing curve and disappearance slope curve of T 1824 in a sheep, and extrapolation from the latter to time of injection (dose of dye = 0·8 mg./kg.).

T 1824 during the first 6 min. after injection in sheep is about 2–3 p.c. of the plasma volume. The more rapid the disappearance rate of the dye used the greater the error. Thus, the error is less with T 1824 than with the red vital dyes.

Hæmatocrit. Although it is theoretically possible to separate completely the cells and plasma by centrifuging, it is doubtful whether this ever occurs. The degree of accuracy to which complete separation is approached determines the degree of accuracy to which the corpuscular volume is measured in any plasma-dye method. This point has not been adequately stressed. In our experience centrifuging sheep blood for 30 min. at 3000 r.p.m. in a centrifuge of 15 cm. radius (one of the

commonest sizes generally employed) fails by 10–12 p.c. to pack completely. It is not possible to pack sheep blood completely with 2760 g. centrifugal force (4000 r.p.m. with 15 cm. radius) within 4 hr., and it requires 18,780 g. (12,000 r.p.m. with 11·5 cm. radius) of centrifugal force for 25 min. to obtain complete packing (Fig. 4). In experiments comparing blood of different species of animals, variations in both centrifugal force and time were found in order to obtain maximum cellular packing [Ponder & Saslow, 1930]. It is necessary to test the validity of any centrifuging method employed.

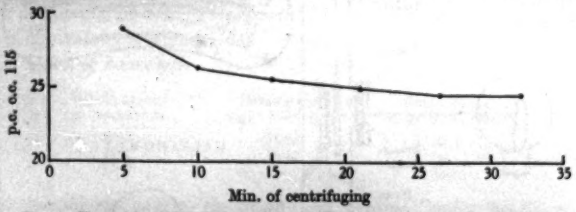

Fig. 4. Sheep blood (adult, heparinized). Time required to pack corpuscles. Centrifuge: 12,000 r.p.m.; 11·5 cm. radius; acceleration 18,780 g.

Description of colorimeter. A very simple type of photocell colorimeter has been used.[1] The light from an incandescent lamp passes through a colour filter, through the trough containing the test solution, and on to the rectifier type photocell, which is connected directly to a pointer galvanometer (Fig. 5). A good deal of latitude in the choice of all the components of the colorimeter except the colour filter would probably cause little error, so long as light intensities on the photocell are kept low. The components actually used are as follows:

Lamp: 6 V., 24 W. car head-lamp bulb supplied by a 6 V. car accumulator. With a suitable lens or reflector much smaller lamps can be used (e.g. a 2 V., 0·6 W. pocket torch lamp).

Colour filter: Wratten No. 29 F. It is important that this filter or a very similar one be used.

Trough and trough holder: Simple, easily made troughs, with a thickness of 1 mm. and a fluid capacity of 0·1 c.c., are made out of 1 mm. bakelite sheet, with microscope cover slips fastened on as sides with wax. The trough is U-shaped, 7 mm. wide and about 15 mm. deep. Troughs of the same thickness must be used for a given series of readings. Each trough must be standardized.

Photocell: Electrocell, Berlin.

Galvanometer: Pointer type, about 10 divisions per microampere. Two scales are provided, a linear one giving readings proportional to the percentage of light transmitted, and a logarithmic one giving readings proportional to the concentration of pigment where Beer's law holds. It is the logarithmic or "concentration" scale which has been used throughout for this work. Where the logarithmic scale is valid, "blank" corrections made with undyed plasma can be subtracted directly from the observed unknown dyed

[1] More particulars of the instrument than are given here may be obtained from the makers, Unicam Instruments, Ltd., Cambridge.

"concentration" reading without any further calculation (see protocol). This gives the dye concentration alone.

A determination is made as follows:

(1) Lamp off; check galvanometer zero.

(2) Lamp on; no trough in place; adjust light intensity till full scale deflexion is obtained =0 on concentration scale.

(3) (*a*) Insert trough containing test solution and take reading on concentration scale.

(*b*) Take reading on dye-free plasma.

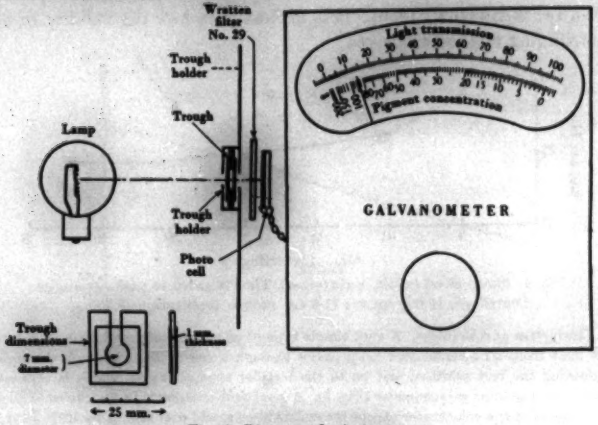

Fig. 5. Diagram of colorimeter.

(4) Subtract 3*b* from 3*a* to obtain reading for dye alone.

(5) If necessary, check reading with known dilution of stock dye.

Sample calculation of blood volume. Data from Fig. 3, using Fig. 2 as calibration curve:

	6 min. sample	9 min. sample	12 min. sample
(1) Observed reading of unknown plasma sample:	31·2	30·8	30·3
(2) Observed reading of dye-free plasma	4·0	4·0	4·0
(3) Corrected reading for dye alone	27·2	26·8	26·3

(4) Calculated extrapolated reading for 0 min. on disappearance curve = 28·1.

(5) Concentration of dye from calibration curve (Fig. 2*b*):

Reading 28·1 = 76·5 p.c. of 1/250 = 1/327 dilution or dilution of dye in plasma at 0 time.

(7) Since 4 c.c. stock dye solution were injected, 4 × 327 = 1308 c.c. = plasma volume.

(8) Hæmatocrit value of dyed sample (Fig. 4) = 24·5 p.c. cells.

(9) Thus, 1308 × 24·5/75·5 = 425 c.c. = corpuscular volume.

(10) Total blood volume = 1308 plasma
425 cells
1733 total

Repeated determinations. For successive determinations on the same animal it is necessary to take account of any dye which remains in the plasma from a previous injection. A blank sample is withdrawn and used in the same way as the dye-free blank in an initial determination.

Errors and controls

(i) *Colour determination.* The usual accuracy may be judged from the calibration values given in Table I. The greatest variation is 0·3 unit, which is equivalent to about 1 p.c. error in plasma volume. The galvanometer needle can be read with accuracy to 0·2 unit over the range used. This is equivalent to about 0·6 p.c. error.

(ii) *Effect of hæmolysis.*

Whole serum no hæmolysis	Same serum slight hæmolysis	Same serum "badly hæmolysed"
33·0	32·95	32·8
19·1	19·15	—
36·0	—	35·8

Slight hæmolysis causes essentially no error; "badly hæmolysed" samples cause an error of about 1 p.c.

(iii) *Overall control.* By adding dye directly to a known volume of blood which is then treated exactly as a withdrawn sample, a "blood-volume" determination can be calculated which includes all the hazards of the method (i.e. dye standardization, colour determination, hæmatocrit reading), except the possibility of specific changes in the dye due to its presence in the living body and errors dependent upon its mixing and disappearance. This is a rigorous test and we strongly recommend it being applied to any dye method as a critical test. The results in the second part of the following table were so obtained:

(i) *Controls testing validity of optical system*

Dye in distilled water		Dye in sheep plasma	
Measured volume c.c.	Calculated volume c.c.	Measured volume c.c.	Calculated volume c.c.
10·0	9·99	100·0	100·3
200·0	201·4	100·0	100·0
—	—	100·0	100·3
—	—	100·0	99·7
—	—	100·0	100·0
—	—	100·0	100·0
—	—	100·0	100·3
—	—	100·0	99·7
—	—	100·0	99·4

(ii) *Controls testing validity of optical system and accuracy of hæmatocrit*

Dye in whole rabbit blood		Dye in whole sheep blood	
Measured volume c.c.	Calculated volume c.c.	Measured volume c.c.	Calculated volume c.c.
50·1	51·2	100·0	100·1
100·0	100·4	100·0	98·9
—	—	100·0	100·0
	—	100·0	102·3

Summary

A method for plasma-volume determination is described, using a simple photo-electric cell colorimeter, a red light filter and a blue dye. The method minimizes the error caused by hæmoglobin and serum pigment and requires small blood samples for measurement, a complete determination being obtainable on 5 c.c. or less. It has several advantages over methods using a visual colorimeter and a red dye. Its accuracy is about 3 p.c.

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J. Physiol. (1938) 93, 285–300 612.817:598.6

NEURO-MUSCULAR CONDUCTION IN THE FOWL

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(*Received* 26 *May* 1938)

Avian muscle has been known for many years to differ in several respects from mammalian muscle, particularly in its high sensitivity to substances having a nicotine-like action. In the course of experiments on these reactions, we found that it differed from mammalian muscle also in its responses to nerve stimulation. We have been unable to find any comprehensive account of the normal behaviour of the avian nerve-muscle preparation, especially in connexion with its action potentials, with which this paper is mainly concerned. The chief differences from mammalian muscle have been found in the effects of initial tension on the twitch response, and in the behaviour of the muscle excited with two nerve volleys. We believe that we have obtained satisfactory evidence to show that certain fibres of an avian muscle require the peripheral summation of two nerve impulses before a propagated disturbance is set up in them. A preliminary account of these experiments has already appeared in this *Journal* [Brown & Harvey, 1938].

Methods

In all the experiments we have used domestic fowls. In most respects white Leghorn hens of 1·5–2 kg. have proved to be most satisfactory, especially for experiments of long duration, as they appear to suffer less from respiratory difficulties. In all experiments anæsthesia was induced and maintained by intravenous injection of 10 p.c. "Pernocton" solution. The initial dose of 0·3–0·5 c.c. per kg. was injected slowly, usually over a period of 5 minutes into a wing vein. Anæsthesia comes on during the injection, and care must be taken to stop the administration as soon as the neck muscles become relaxed. The margin between full anæsthesia and a fatal stoppage of respiration is narrow, and the respiratory movements must be watched with care during the administration of the anæsthetic. The initial dose maintains anæsthesia for about $\frac{3}{4}$ hr., after

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which further injections of approximately 0·1 c.c. per kg. may be made into a cannula in the jugular vein whenever necessary. Capons are to be avoided, as they are extremely sensitive to the anæsthetic. In the experiments in which curarine was employed, the animals were first anæsthetized with "Pernocton" and the muscles prepared for recording. Immediately before the administration of curarine, a string was tied tightly round the neck, just below the head, artificial respiration was started, and the brain and medulla were destroyed by thrusting a bradawl into the foramen magnum.

The muscle used for recording in the majority of the experiments was the lateral head of gastrocnemius, which was prepared as follows. The feathers were plucked from the leg and thigh, and the skin was incised along the posterior aspect of the leg from the tarsus to the upper end of the femur. The tendon of gastrocnemius was detached just proximal to the tibio-tarsal joint, and the external head of the muscle was separated from the middle head and from the muscles covering the tibia, as far as the knee joint. The vastus externus and the semimembranosus and semitendinosus were retracted, exposing the sciatic nerve and blood vessels which lie on the posterior surface of the femur. The transversely lying muscle fibres, which cover the nerve and vessels, just behind and above the knee joint, were carefully incised and the nerve and vessels dissected from each other. The nerve was cut some 5 cm. proximal to gastrocnemius, and all branches, other than the twig supplying the lateral head of gastrocnemius, were cut.

The muscle and nerve were now covered with hot moist cloths, and drills were inserted in the upper end of the femur, the condyles of the femur and into the lower end of the tibia. These provided a means of fixing the leg rigidly to the myograph base. The preparation was completed by tying a steel hook, with an insulating segment, into the tendon.

The methods used for stimulation and recording were the same as those previously described for cats [see Brown & Euler, 1938, and previous papers from this laboratory]. In a number of experiments, we have recorded the electrical responses of a muscle excited by directly applied induction shocks. For this purpose, the gastrocnemius could not be used, as we found it impracticable to excite such a large muscle maximally with a single shock. We therefore used the flexor perforatus indicis secundus pedis, a flattened muscle about 3 cm. long, which lies beneath the origin of the external head of gastrocnemius, and receives a branch from the nerve to gastrocnemius. The stimuli were break induction shocks from coreless coils, and were applied to the muscle by silver pins

thrust into its substance. The action potentials were led by small calomel half cells to a two-stage D.C. amplifier and thence to a cathode-ray oscillograph.

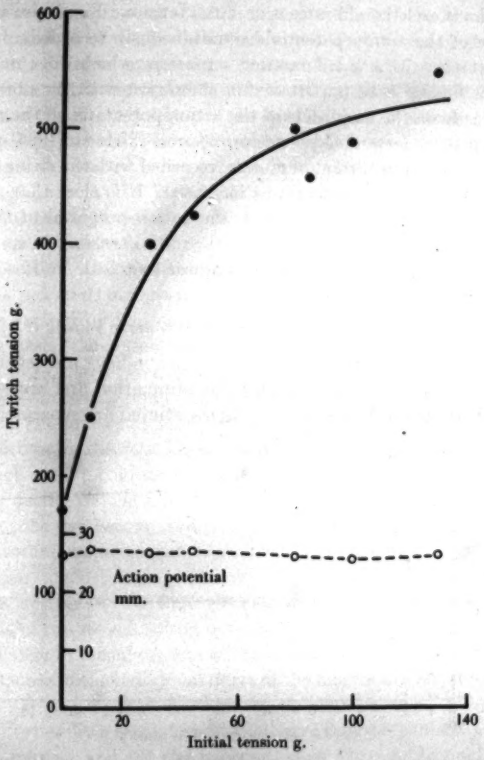

Fig. 1. Curves showing effect of initial tension on twitch tension (●) and action potential (○) of fowl's gastrocnemius.

RESULTS

(a) *The effect of initial tension on the isometric twitch*

The tension response of the fowl's gastrocnemius to single maximal motor nerve volleys is profoundly influenced by the initial tension applied to the muscle. Fig. 1 illustrates this phenomenon. With the initial

tension zero, the twitch tension was 170 g. Increase of the initial tension to 130 g. caused an increase in twitch tension to the high value of 550 g. These changes are many times greater than those produced in frog's and mammalian muscle by alterations in initial tension. Fig. 1 also shows the amplitude of the action potentials simultaneously recorded. It will be noted that there is a small random variation, which does not exceed 5 p.c. This finding is of importance in connexion with the observations we have made on the amplitude of the action potentials of the responses to two successive nerve volleys (*vide infra*), since Fulton [1926] described increase in the action current of muscle, recorded with the string galvanometer, when the initial tension was increased. It is clear that, with the methods of recording we have used, the action potential of the fowl's muscle is not significantly affected by the initial tension, in spite of the great effect on the tension of the mechanical twitch.

(*b*) *Response of the muscle to two nerve volleys*

The muscular summation curve. When two volleys are set up at the same point in the motor nerve, muscular summation first appears when the stimuli are 0·8–1·0 msec. apart. As the stimuli are separated in time

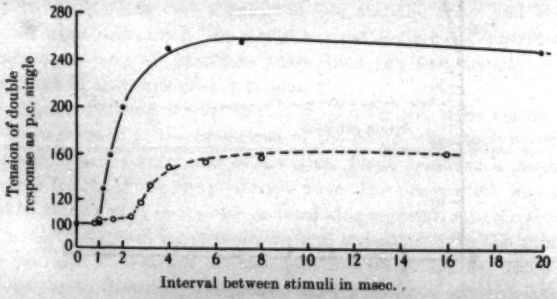

Fig. 2. Curve showing summation of muscular response to two nerve volleys in the fowl's gastrocnemius (●) and cat's tibialis anterior (○).

the tension curve rises very steeply, and at 1·2 msec. the combined tension may be 250 p.c. of the single response (Fig. 2). Further separation of the stimuli yields only a very small increase in tension, the maximum tension being reached with an interval of 4–5 msec. This description applies only to fresh muscles; as an experiment proceeds, the

curve relating summated tension to stimulation interval is flattened at the shorter intervals, and slowly reaches its maximum at an interval as long as 10 msec. In the same figure is shown a similar muscular summation curve derived from the cat's tibialis anterior. The ratio of the tension of a double response of the bird's muscle to the tension of a single response has varied in different experiments between 2·4 and 3·7. These values are greater than those normally found in mammalian muscle, with the exception of the internal rectus of the eye [Cooper & Eccles, 1930]. The difference is probably due largely to the effects of initial tension, described above, since the second volley excites fibres which are already beginning to develop tension. We believe, however, that this does not entirely account for the high ratio, and that the entry into contraction of additional fibres, which have not responded to the first volley, as described in the next section, also contributes to the effect.

Action potentials accompanying the responses to two volleys. When the muscle is excited by two maximal nerve volleys set up at intervals less than 150 msec., the action potential accompanying the second muscular response is regularly greater than the first. Fig. 3 shows a series of responses at intervals of 18, 40, 90 and 200 msec., and Fig. 4 shows the results of a similar experiment plotted graphically. As soon as the action potentials become discrete, i.e. at about 20 msec. interval, the second may be as much as 25 p.c. greater than the first. With further separation, the disparity declines along a smooth curve, and, at intervals greater than 150 msec., the two become and remain equal. This difference between the first and second responses can be seen with tetanic stimulation at suitable frequencies.

There appear to be four possible explanations of the increased amplitude of the second action potential: (*a*) increase in action potential due to change in initial tension of the muscle; (*b*) closer synchronization of the response of the individual fibres of the muscle as a result of previous activity; (*c*) a property inherent in the muscle fibre, analogous to "Treppe" whereby a second excitation wave is accompanied by a greater action potential; and (*d*) the contraction of fibres additional to those excited by the first volley. The possibility that local effects in the nerve are responsible for the phenomenon has been excluded by delivering the stimuli, in some experiments, through two separate pairs of electrodes, some 2 cm. apart on the nerve. The first volley was set up by the pair distal to the muscle and the second through the proximal pair. Under these conditions the phenomenon occurs just as well as when both volleys are set up through the same electrode.

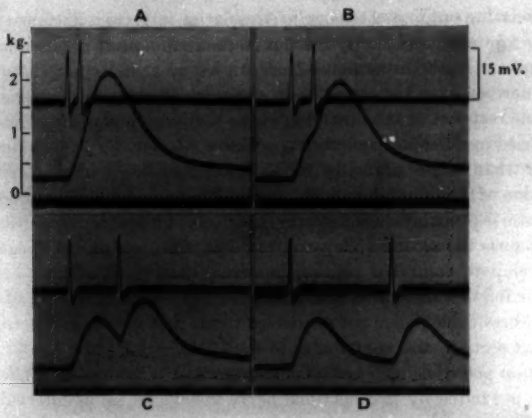

Fig. 3. Hen 1·9 kg. Myograms and action potentials of gastrocnemius in response to two maximal nerve volleys (A) 18, (B) 40, (C) 90 and (D) 200 msec. apart. Time 10 msec.

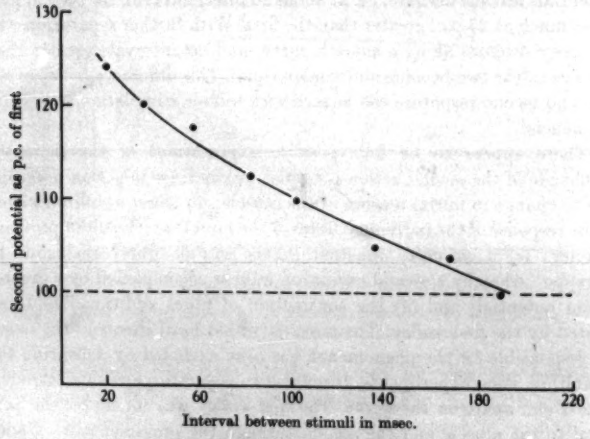

Fig. 4. Curve showing relation between size of second action potential and interval at which it follows the first potential.

(*a*) This explanation is adequately excluded by the experiments described above, in which increases of initial tension, nearly as great as the tensions evoked by a single nerve volley, were accompanied by changes in action potential not greater than 5 p.c.

(*b*) We have no evidence that the second response shows less temporal dispersion than the first; in fact, the second potential wave is often of longer duration than the first. Fig. 5 shows two responses of the flexor perforatus muscle to two nerve volleys 10 msec. apart; the second response is 21 p.c. greater in amplitude than the first and 17 p.c. greater in duration.

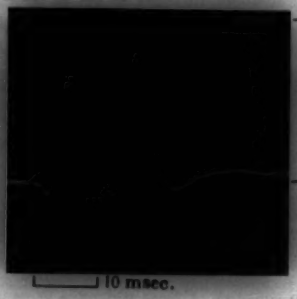

Fig. 5. Hen 2·5 kg. Action potentials of flexor perforatus muscle in response to two maximal nerve volleys 10 msec. apart. D.C. amplifier and cathode-ray tube.

(*c*) When avian muscle is excited by directly applied induction shocks, the second response is not greater than the first. Fig. 6 shows (A) the responses of the flexor perforatus muscle to two stimuli through the nerve; (B) to two stimuli applied directly to the muscle 1 cm. distant from the recording electrodes; and (C) to two stimuli applied 2 mm. from the recording electrodes. The response to nerve stimulation shows the characteristic difference in the amplitude of the action potentials, the second response being 12 p.c. greater than the first. When the stimuli are applied directly to the muscle, but at some distance from the recording electrodes, the fibres from which the action potential arises are mainly excited through the nerve, since the fibre length in this muscle is not more than 8 mm. Under these conditions, the difference between the first and second action potentials is still evident. In Fig. 6 B the second response is still 6 p.c. greater than the first. When, on the other hand, the stimuli are applied sufficiently close to the recording electrodes for

the excitation to include all the fibres from which the recording electrodes are leading, the second response is only 93 p.c. of the first, Fig. 6C. Since the stimulation artefact is of almost equal magnitude in B and C it is unlikely that any essential differences in size between the first and second responses have been obscured by it.

(*d*) We are therefore left with the probability that the increase of the second action potential is due to the contraction of additional muscle fibres. It would appear that certain fibres of the bird's muscle cannot be excited by a single nerve impulse, and that such an ineffective impulse leaves behind it a period of raised excitability of the muscle end plate, which enables a second incident impulse to act as an effective stimulus.

In connexion with the experiments on direct stimulation of muscle,

Fig. 6. Hen 2·5 kg. Action potentials of flexor perforatus in response to two stimuli 10 msec. apart applied A to the nerve, B to the muscle 1 cm. from recording electrodes, and C to the muscle, 2 mm. from recording electrodes. D.C. amplifier and cathode-ray tube.

we attempted to record the electrical response of directly stimulated muscle after degenerative section of its motor nerve. The mechanical response of such a muscle is noticeably slower, in all its phases, than that of the innervated muscle when directly stimulated; this slowing is associated with a very great temporal scattering of the electrical response. So great is this dispersion, that the action potential consists of an irregular and inconstant wave, many times longer in duration than the normal potential, and consequently completely unsuitable for accurate measurement of its amplitude.

In most of the experiments on the effects of two volleys, the muscle has been covered with a cellophane bag and warmed by an electrical heater with a bowl reflector, placed about 1 metre away. Under these conditions, and with the muscle in the vertical position for recording, its temperature is materially lower than in the body. In one experiment,

therefore, we enclosed the whole bird in a heating cabinet, and measured the muscle temperature with a thermocouple. Increasing the muscle temperature from 29 to 36° C. did not produce any significant change in the ratio of the first to the second electrical response to two volleys 25 msec. apart. The normal temperature of the leg muscles in the unæsthetized hen is, indeed, approximately 40° C., and we did not succeed in raising the temperature of the exposed, experimental muscle to this level. But we regard the fact that, with increase of the muscle temperature from 29 to 36° C., the disparity between the electrical responses to the two stimuli remains unaltered, as a strong indication that it represents a normal characteristic of the muscle, under natural conditions.

Response to tetanic stimulation. The frequency of tetanic stimulation producing a fully fused tension response in the fowl's muscle is of the order of 60 per sec., a frequency not greatly different from that required by corresponding muscles of the mammal. As might be expected from the summation curve with two volleys, the ratio of twitch tension to full tetanic tension is high, in the region of 8. Cooper & Eccles [1930] give values between 3·26 and 4·95 for this ratio in mammalian muscles. The internal rectus of the eye, with a twitch-tetanus ratio of 10·7, is the only mammalian muscle which has a ratio resembling that found for avian muscle.

As mentioned above, the action potentials of avian muscle during a tetanus show the disparity between the first and second responses observed with two volleys. At frequencies of stimulation of 50 per sec., when the potentials are discrete, the second action potential is regularly larger than the first; the third and subsequent responses are of the same size as the second, until the decline develops, which normally ensues in a prolonged stimulation. The diminished responses at the end of a long tetanus are considerably prolonged, and their small amplitude is probably attributable to temporal dispersion of the responses of the individual fibres.

Effect of a preceding tetanus on the response to one and two volleys. In the bird, as in the mammal [cf. Brown & Euler, 1938], a tetanus evokes a long lasting increase in the twitch tension Fig. 7. It will be noted in this figure that the responses to the single volleys which precede the tetanus are somewhat irregular. This phenomenon we have observed in most experiments, and figures given by van Dijk [1934] show the same effect. A similar irregularity has been seen in the tension response optically recorded, and in the action potential with single volleys. Such an irregularity is to be expected if a single volley fails to excite all the fibres of the

muscle, since very slight variations in the thresholds of individual fibres will throw them in or out of action, in the responses to successive volleys. The potentiated twitches immediately succeeding the tetanus show, on the other hand, a regular decline, the usual irregularities of twitch tension being absent during the period of potentiation. This suggests that a tetanus is followed by a period of raised excitability of the motor end plates, like that observed in the partially curarized mammalian muscle, which enables a single nerve volley to excite all the muscle fibres. It was, accordingly, to be expected that the normally observed increase in the

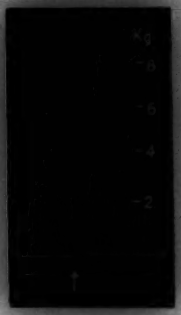

Fig. 7.

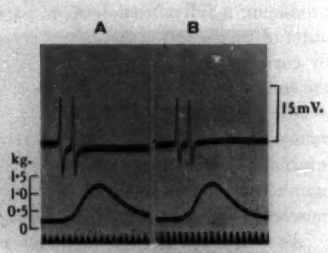

Fig. 8.

Fig. 7. Hen 1·6 kg. Tension response of gastrocnemius to maximal nerve volleys at intervals of 10 sec. At arrow, motor nerve tetanus at 50 per sec. for 10 sec.

Fig. 8. Hen 1·5 kg. Myograms and action potentials of gastrocnemius in response to maximal nerve volleys 20 msec. apart, A, before and B, 280 msec. after a tetanus at 30 per sec. for 1·0 sec. Time, 10 msec.

second response to a pair of stimuli would, after a tetanus, be absent. This proved to be the case. Fig. 8 shows the electrical responses of the fowl's gastrocnemius to two maximal volleys, 20 msec. apart, A before, and B 280 msec. after a tetanus at 30 per sec. for 1·0 sec. In the original record, the negative deflexion of the first response before the tetanus was 12·7 mm., and of the second 14·7 mm., a difference of 16 p.c. After the tetanus, the first response was 12·8 mm. and the second 12·3 mm. The tetanus has, therefore, abolished the normal enhancement of the second response, and replaced it by a small (4 p.c.) diminution.

We stated above that the increase size of the second response was probably attributable to the entry into contraction of additional fibres, which did not respond to the first volley, and we suggested that a tetanus

would lower the threshold of all the muscle end plates for a longer period, so that the first volley, following within a few seconds after a tetanus, should excite all the muscle fibres. On this assumption, it might be expected that the first electrical response to a pair of volleys after a tetanus should have the same amplitude as the second to the pair immediately preceding the tetanus. In the experiment quoted above, however, the first action potential of the post-tetanic pair is no greater than the first of the pre-tetanic pair, suggesting, at first sight, that the first post-tetanic volley is still, in part, subliminal, exciting only about the same number of muscle fibres as the first pre-tetanic volley. We are convinced that such an interpretation would be incorrect, and that the amplitude of the electrical response to the volley following the tetanus is reduced, not because all fibres are not responding, but by the interference of another effect. Brown & Euler [1938] have recently shown that the potentiated twitches, which follow a tetanus in mammalian muscle, are accompanied by smaller action potentials than the normal twitches preceding the tetanus, and that this diminution in action potential is the result of a depolarization of the muscle, and not a consequence of a fall in the number of muscle fibres responding. It could in any case be regarded as probable that this reduction of the post-tetanic electrical response by depolarization would occur in avian as in mammalian muscle. There is, moreover, evidence that it does so. Table I shows the changes

TABLE I

Action potentials before tetanus (mV.)		2nd as p.c.	Frequency tetanus	Action potentials after tetanus (mV.)		2nd as p.c. 1st after
1st	2nd	1st	per sec.	1st	2nd	tetanus
22·7	25·8	114	3	21·7*	24·2*	112*
21·6	25·7	119	30	22·1	21·1	96
23·1	26·3	114	50	21·7	21·7	100
21·2	25·7	122	240	21·3	22·1	104
22·4	25·6	114	600	21·7	21·7	100
22·2	25·8	117		21·7	21·7	100

Diminution of 1st produced by tetanus 2 p.c.
,, 2nd ,, 16 p.c.

* These figures are omitted from mean values of post-tetanic pairs as the tetanus was of too low a frequency to produce its characteristic effect.

produced by tetanus in the electrical responses to pairs of stimuli. We have reason to believe, as stated above, that the second response of a pair represents a contraction of all the fibres of the muscle; if, therefore, the second responses of pairs are compared before and after a tetanus they should give a clear indication of the degree of depression of the total action potential by the tetanus, uncomplicated by any change in the number of

fibres involved in the response. Thus, in Table I, the second responses of post-tetanic pairs are 16 p.c. less than the second responses of the pre-tetanic pairs. At the same time, the *first* responses of the post-tetanic pairs are only 2 p.c. less than the corresponding first pre-tetanic responses. This suggests that the tetanus has increased the number of fibres responding to the first volley by 14 p.c. Before the tetanus, the second responses were 17 p.c. greater than the first responses; after the tetanus

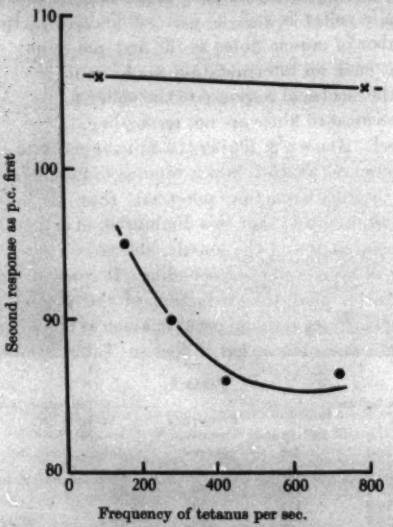

Fig. 9. Curve showing relation between frequency of tetanus and size of second response of a subsequent pair of responses 45 msec. apart (●). The size of the second responses before the tetani are shown above (×).

the two responses are approximately equal. It would appear, therefore, that the tetanus has so increased the first response that it involves nearly all the fibres of the muscle, but the increased action potential which would normally accompany the entry into activity of these additional fibres has been masked by the 16 p.c. reduction in the total action potential, produced by the post-tetanic depolarization. We conclude, therefore, that a tetanus is followed by a short period of raised excitability of the motor end plate, which enables a single nerve impulse to excite fibres which

previously required the incidence of two impulses to bring them into activity.

It will be noted in Table I that the second of the pair of responses after the tetanus at 30 per sec. is only 96 p.c. of the first. In certain experiments this effect has been very prominent and the second response after the tetanus has been regularly less than the first. There appears, moreover, to be a relationship between the frequency of the tetanus and the degree of depression of the second response. Fig. 9 shows graphically the results of one experiment. Before tetanic stimulation, the second response of a pair 40 msec. apart was between 5 and 7 p.c. greater than the first. After tetanization at 180 per sec. the second response was 5 p.c. less than the first and the difference between the two increased as the frequency of the tetanus increased. The significance of this finding will be considered in the discussion.

Effect of drugs on response to two volleys. On the supposition that adrenaline might exert some influence on muscle end-plate excitability, we studied in a few experiments the effect on the response to two volleys of adrenaline administered by intravenous injection. In doses up to 150 γ per kg., adrenaline has very little effect on the disparity between the action potentials in response to two volleys, 25 msec. apart. There was a suggestion that the responses approached a little nearer to equality through increase in the size of the first response, but the change was inconstant and of doubtful significance. It is possible that stimulation of the sympathetic supply of the muscles might have yielded more conclusive results, but we found that such stimulation was not feasible in the bird.

As might be expected, from its effects in the mammal [Brown, 1938], curarine, in doses insufficient completely to block neuro-muscular conduction, greatly exaggerates the disparity between the action potential responses to a pair of nerve volleys. Thus, in one experiment, on the flexor perforatus muscle, the second action potential of a pair set up 10 msec. apart was 9 p.c. greater than the first before curarine; after intravenous injection of 1 mg. of curarine the second potential was 68 p.c. greater than the first.

Discussion

The experiments on fowl muscle described above have revealed certain very characteristic differences between its responses to nerve stimulation and those of the muscle of mammals. The large effects of initial tension on the twitch tension are probably associated with the particular postural and phasic functions of the muscle we have examined, and we are

unaware whether they apply to other muscles of the bird. The initial tension effect can introduce a very serious source of error into experiments on these muscles, and must be controlled with exactitude if reproducible responses are desired.

The most striking difference between avian and mammalian muscle is seen in their electrical responses to two closely following maximal nerve volleys. In mammalian muscle such responses are equal; in other words, no more muscle fibres are excited by the second volley than by the first. In the bird, the second response is greater, not only in amplitude, but in duration. The neuro-muscular transmitting apparatus is certainly involved in this effect, since the electrical responses of muscle directly excited with two maximal shocks are equal, or, when a difference occurs, the second is smaller in amplitude than the first. We have excluded other possible causes of the phenomenon, and are left with the probability that the increase in the second action potential is due to the fact that the second volley has excited additional muscle fibres, which failed to respond to the first volley. Complete proof of this assumption could be provided only by showing that the additional fibres of the muscle could respond to a directly applied stimulus, during the refractory period of the nerve and of the fibres responding to the first volley. The difficulty of detecting a small increment of potential, masked by a large stimulus artefact and by the temporal dispersion among the fibres of a fairly large muscle, which alone precludes accurate timing of the second stimulus, has, however, so far prevented us from demonstrating this.

The phenomenon of the augmented second action potential has been observed by Samojloff [1908] and by Adrian & Lucas [1912] in freshly isolated frog's muscle, stimulated through its nerve, and it appears to differ only in its time relations from the effects we have observed. In the fowl, as in the frog [Bremer, 1927], the effect is greatly exaggerated by small doses of curarine, the use of which substance is necessary in the mammal to elicit the effect at all [Brown, 1938].

The effect of a tetanic stimulation in producing a longer increase in end-plate excitability, causing the first response to a post-tetanic volley to approach maximality, is obviously very closely allied to the relief of a partial curare paralysis by a similar tetanus [Boyd, 1932; Brown & Euler, 1938].

In the description of the experiments on the effects of tetani we have referred to the difficulties of interpretation of the size of the action potentials following a tetanus, on account of the reduction in the amplitude of a maximal electrical response, accompanying the post-tetanic

mechanical potentiation. In the experiments shown in Table I this effect of a tetanus could be controlled by comparing the sizes of the second responses before and after the tetanus, on the assumption that they were maximal responses both before and after. A further complication is, however, introduced in some experiments by the finding that the second response after the tetanus is significantly smaller than the first, as shown in Fig. 9. This suggests, at first sight, that the second response has now become submaximal, in comparison with the now maximal first response. The validity of such an interpretation is, however, made doubtful by the fact that the second response of *directly* excited muscle is often less than the first (Fig. 6C). A similar reduction in the size of a second directly excited response has been described for frog's muscle by Adrian & Lucas [1912]. It would appear then that in amphibian and avian muscle, at least, the electrical change of a second response is less than the electrical change of a first response, *if the first response is maximal*. In the case, therefore, of the experiments referred to in Fig. 9, we must conclude that the depression of the second responses after a tetanus is attributable to the fact that they follow first responses, which have approached closer to maximality as the frequency of the preceding tetanus has increased. The cause of this depression of the action potential of a second response, following a maximal first response, is open to question. It is unlikely that it can be due to persisting refractoriness of fibres excited by the first stimulus, since in frog's muscle the depression may still be present at an interval of 50 msec., and in the fowl at an interval of 40 msec. A prolonged negative after-potential has been found by Brown & Euler to follow a tetanus, and this is accompanied by a reduction in subsequent single action potentials. The single response of frog's muscle is accompanied by a slow negative after potential which lasts, according to Schaefer [1936], some 50–80 msec. Adrian & Lucas [1912] show that the reduction in second response of directly stimulated muscle also lasts about 50 msec. (their Fig. 10). It seems, therefore, not unreasonable to attribute the reduction in second action potential to its falling in the period of negative after-potential following the first response. Normally in avian muscle, this reduction is completely obscured by the failure of the first volley to excite all the muscle fibres and by the increment of fibres participating in the second response.

We conclude that about 25 p.c. of the muscle fibres of the fowl's leg muscles fail to respond by contraction to a single nerve impulse. The ineffective impulse leaves behind it at the motor end plate a period of raised excitability which lasts some 150 msec., and a second impulse

reaching the motor end plate during this period is now effective in setting up a contraction. A period of tetanus leaves behind it a similar, but longer lasting period of enhanced excitability, during which single nerve volleys can excite all the fibres of the muscle, though the interference of the prolonged depolarization due to the tetanus prevents a corresponding enhancement of the electrical response.

Summary

1. Optical isometric myograms and action potential records have been taken from the leg muscles of the domestic fowl anæsthetized with pernocton.

2. The initial tension to which the muscle is subjected has a much greater effect on the maximal motor nerve twitch tension than in the mammal.

3. When two maximal motor nerve volleys follow one another at intervals less than 150 msec., the action potential in response to the second is greater than the first.

4. This effect has been shown to be due to the fact that a single nerve volley fails to excite all the fibres of the muscle, but leaves behind it a period of raised excitability of the motor end plates. A second volley arriving during this period can thus excite the fibres which failed to respond to the first volley.

5. Brief tetanic stimulation of the motor nerve is followed by a long lasting increase in twitch tension and an increase in excitability, which enables a single subsequent volley to excite the muscle maximally.

6. Certain difficulties in the interpretation of changes in the amplitude of action potentials are considered in relation to the depolarization which follows a tetanus, and the slow potentials accompanying a single twitch.

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J. Physiol. (1938) 93, 301–304 612.141

A PLETHYSMOGRAPHIC METHOD FOR MEASURING SYSTOLIC BLOOD PRESSURE IN THE INTACT RAT

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(*Received* 3 *June* 1938)

By means of the technique described in this paper it is possible to make repeated measurements of the systolic blood pressure of the rat without injury to the animal.

Principle. The circulation through the tail is arrested by suddenly inflating a cuff on the root to a pressure well above the systolic level. The pressure is then gradually reduced. When the systolic level is reached, blood can enter but cannot leave the tail. The resulting increase in the volume of the tail is detected by a simple water plethysmograph.

Apparatus. The cuff (Fig. 1) consists of an outer cylindrical casing of celluloid (X-ray film) about 2·5 cm. long by 2 cm. diameter, and an inner tubular sheath of very thin condom rubber which closely invests the tail. The sheath is held at each end between an inner collar of glass tubing (0·5 mm. thick) and an outer disc of cork, the periphery of which is fixed to the outer casing. The glass collars and the rubber sheath have the same internal diameter. The length of sheath in contact with the tail should be at least 1·5 cm. An air inlet tube of narrow glass tubing is cemented into the outer casing and is attached by bicycle valve tubing to a three-way tap, through which the cuff can be connected with the outside air or with a pressure reserve bottle of about 500 c.c. capacity. The bottle is also connected with a mercury manometer and with a sphymomanometer bulb and release valve. Several cuffs must be constructed to fit tails of different thickness. They should be stored in a refrigerator and discarded as soon as the rubber begins to perish.

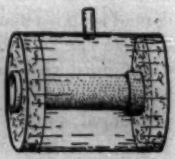

Fig. 1. The cuff.

[1] In receipt of a full-time grant from the Medical Research Council.

The plethysmograph (Fig. 2) is a glass tube about 12 cm. long and about 1 cm. internal diameter, terminating in a capillary tube of about 0·5 mm. bore, and bearing a side tube for filling and for adjusting the level of fluid in the capillary. Junction between the tail and the plethysmograph is made by green soft soap contained in a screw-capped metal gland.[1] This junction is watertight, but does not obstruct the venous return unless the soap is too hard.

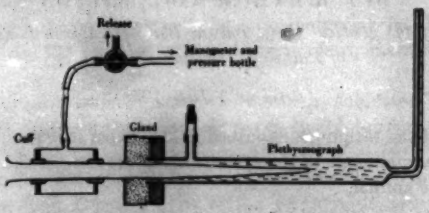

Fig. 2. The plethysmograph and cuff mounted on the rat's tail.

Procedure. A suitable cuff is fitted on the tail about 1 cm. distal to the root. The accuracy of the method depends mainly on the fit of the rubber sheath, which should be just loose enough to allow free rotation of the cuff round the tail. The cap of the plethysmograph gland is removed, packed with soft soap and replaced with the thread lightly engaged. A central core of soap is removed by a narrow bladed knife and the tail is inserted through the hole to within about 2 cm. of the cuff. The cap is screwed down until the tail is felt to be lightly gripped by the soap. The plethysmograph is filled with lukewarm distilled water, coloured with phenol red, and the level in the capillary adjusted to a convenient point. The column rises and falls slightly with respiration and finer and more rapid pulse waves are often visible. Before attempting a reading it is desirable to make sure that the venous return from the tail is not obstructed by the gland or the cuff. The venous pressure in the tail is normally so low that inflation of the cuff to a pressure of 10–20 mm. Hg is enough to compress the main veins and cause a steady rise of the meniscus in the capillary tube. If a greater pressure is needed either the cuff is too tight, the soap is too hard, or the cap has been screwed down too tightly.

[1] The brass case supplied with a microscope objective can readily be converted for this purpose.

To take a reading the air bottle is filled to a pressure of 200–250 mm. Hg and then connected with the cuff through the three-way tap. The meniscus rises abruptly as blood is milked backwards from beneath the sheath of the cuff and, if necessary, the level is readjusted. The pressure is next reduced in 10 mm. stages through the release valve. The meniscus remains stationary or falls slowly until the systolic level has been passed, when an abrupt and progressive rise is observed. The manometer reading is noted and the air in the cuff is released through the tap. The fluid column falls rapidly as blood leaves the tail. When equilibrium has been regained a second and more accurate reading is taken, the pressure being reduced in 2 mm. stages as the expected systolic level is approached. The end point is sharp.

Applications and limitations. The chief application of the technique is in experiments demanding serial readings of blood pressure at intervals over a period of weeks, e.g. in the study of experimental hypertension. For single experiments, such as testing fluids for pressor activity, direct cannulation of the carotid is more suitable. Since the slightest movement of the animal is transmitted to the plethysmograph, anæsthesia is essential. Ether has been found to be the most reliable anæsthetic provided that the concentration of vapour during induction is not excessive. Too strong an atmosphere of ether stimulates bronchial secretion which may obstruct respiration seriously enough to affect the blood pressure or even cause death. If such obstruction should occur it can generally be relieved by a suction tube inserted into the pharynx, but blood pressure readings are of little value until cyanosis has disappeared. Experience with a direct method of recording blood pressure through a carotid cannula has shown that with ether anæsthesia the pressure can be maintained within the normal range for two hours or more. Chloroform causes a pronounced fall in pressure. Diallylbarbituric acid ("Dial", Ciba) injected into the peritoneal cavity has also been used and has given results comparable to those obtained with ether. Dial anæsthesia, however, generally lasts for two hours or more, during which time the animal must be carefully observed for signs of respiratory obstruction.

Accuracy. In a series of 22 rats direct determination of systolic blood pressure was made by inserting into the carotid artery a No. 12 gauge needle connected with a mercury manometer [Rous & Drury, 1929], coagulation being prevented by preliminary intravenous injection of chlorazol fast pink [Huggett & Rowe, 1933]. Repeated readings of systolic pressure in the tail were then taken and compared with the carotid pressure. It was found that with badly fitting cuffs the two estimates

might differ by 20 mm. Hg or more. With properly fitting cuffs, however (18 out of 22 experiments), the present technique was found to give readings consistently lower than the carotid value by 10 mm. Hg or less.

Normal range. In Table I data obtained from 88 normal rats, mostly young adults, are collected, analysed, and compared with the estimates

TABLE I

Author	Method	Anæsthetic	No. of observations	Blood pressure		
				Mean	Range	Standard deviation
Durant [1927]	Direct	Ether and amytal	41	119	92–150	—
Rous & Drury [1929]	,,	Urethane	?	105	100–120	—
Chanutin & Ferris [1932]	,,	Ether	10	120	106–145	—
Leiter [1935]	,,	Sodium barbitol	69 p.c. of 131	—	101–140	—
Rubin & Rapoport [1937]	Indirect (leg)	Ether	117	119	78–154	19
Griffiths [1934]	Indirect (leg)	Nembutal	90 p.c. of more than 100	—	80–100	—
Present method	Indirect (tail)	Ether	70	106	78–132	13·0
,,	,,	Dial	18	105	87–122	11·4

of other writers. Taking three times the standard deviation from the mean value of 106 as a safe margin, readings consistently above 145 or below 65 mm. Hg may be regarded as abnormal.

SUMMARY

A method is described for measuring the systolic blood pressure of the intact, anæsthetized rat using an air pressure cuff and a plethysmograph on the tail. In normal rats anæsthetized with ether, the method gives results slightly lower than the carotid blood pressure, ranging from 78 to 132 mm. Hg with a mean value of 106 mm.

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J. Physiol. (1938) 93, 305-311 612.745.3:612.741.15

THE RELATION OF CONTRACTURE TO THE INCREMENT IN THE RESTING HEAT PRODUCTION OF MUSCLE UNDER THE INFLUENCE OF POTASSIUM

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(*Received* 9 *May* 1938)

It has been shown that the heat production of the sartorius muscle of the frog is increased by potassium chloride in concentrations of four to ten times that found in normal Ringer's solution [Solandt, 1936]. This increment was considered to be independent of any shortening process and was described as an increase in the resting metabolism. In higher concentrations there was no liberation of heat in excess of normal unless a contraction occurred. Hegnauer *et al.* [1934], on the other hand, state that the rate at which oxygen is taken up by the muscle under the influence of potassium is only increased by concentrations which give rise to shortening. In view of these findings it appeared advisable to extend the study of the changes in heat production due to potassium in order to determine the possible relationship between increased metabolism and mechanical changes induced in the muscle.

Method

The rate at which heat was liberated by the muscle was determined in the manner described by Solandt [1936]. The double sartorius preparation from frog (*Rana pipiens*—Canadian) was used throughout. This was mounted on a Downing-Hill muscle thermopile which was then enclosed in a brass chamber. It was set up so that the muscle supported a light lever by a thread which passed through a tube fixed in the upper part of the instrument. The latter was immersed in a water bath at 29·4° C., and arrangements were made for running solutions in and out of the chamber from glass containers kept in the same bath. As the

solutions were thus always at bath temperature they did not upset the thermal equilibrium of the thermopile system to any marked extent.

The flow of current set up in the thermopile circuit due to the heat liberated by the muscle was recorded by a d'Arsonval moving-coil galvanometer. The millimetre scale on which the deflexion of the reflected beam was recorded was calibrated in absolute units of heat. This was done by passing condenser discharges through filter paper (soaked in Ringer's solution) which was substituted for the muscle.

In performing an experiment the apparatus was set up as described, Ringer's solution was run into the chamber and kept aerated with oxygen. At the end of 1 hr. the solution was removed, and after 2 min. a reading of the resting heat was taken. The Ringer's solution containing an increased amount of potassium was now added, left for 3 min. and then removed. A reading was again taken 2 min. after removal. The procedure was repeated at regular intervals during the period of the experiment.

The Ringer solution used as normal contained 0·625 g. NaCl, 0·015 g. KCl, 0·020 g. $CaCl_2$ made to 100 c.c. with distilled water. The amount of potassium was increased as desired by adding a calculated amount of isotonic aqueous KCl solution.

The mechanical changes induced in the muscle by the concentrations of K studied (1–60 times that of normal Ringer's solution) were determined in an independent series of experiments. The procedure here was to set up a double sartorius preparation horizontally in Ringer's solution with just sufficient tension applied to keep it extended. Using a dissecting microscope a suitable blood vessel in the muscle was sharply focused and marked by a cross-hair in the eyepiece. In this way when a solution of increased potassium concentration was added the slightest amount of shortening could be observed directly, and twitching of single fibres could be detected.

Results

Fig. 1 shows the changes in the rate at which heat is liberated by muscle during the time it is exposed to solutions having potassium concentrations of 7, 8, 9, 10, 12 and 22 times that of normal Ringer. The observed galvanometer deflexions at intervals of 6 min. are plotted for a K concentration of 7 times normal, as an example of the regular rise and fall obtained in these experiments. Although each curve shown was the result of one experiment, essentially the same results were obtained on at least three occasions for each K concentration represented. The plotted results show that the resting heat production was increased

by potassium. This was true for all K concentrations from 4 to 60 times that of normal Ringer. There was a subsequent depression in heat production which was marked in the case of the solutions rich in K. For the solutions with K concentrations less than 12 times normal the resting heat production did not return to a normal value during the experiment (5 hr.) although there was a steady retreat from the maximum in all cases.

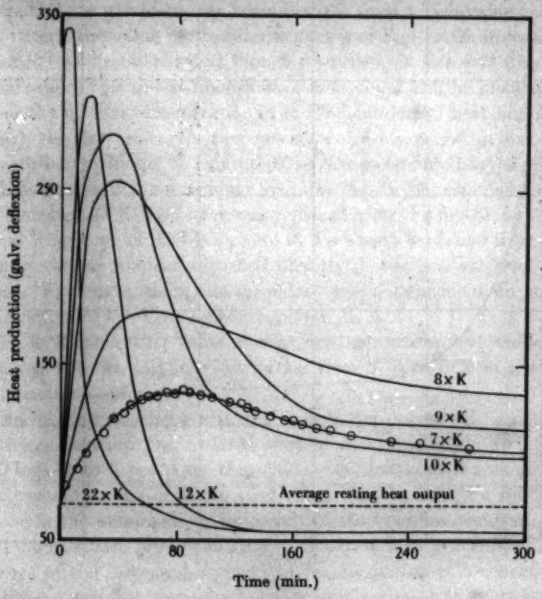

Fig. 1.

For concentrations of K from 4 to 6 times that of Ringer the effects were variable. There might be a slow steady rise during the 5 hr. of the experiment or a maximum might be attained before that time. Occasionally no rise took place for a variable initial period, after which an abrupt rise to a maximum would occur. For concentrations over 6 times normal, the responses were more consistent as represented in Fig. 1. From these curves it is evident that the increase in heat production proceeds more and more rapidly as the concentration is raised, and that

at the same time the subsequent fall is hastened. Fig. 2 shows how the time to reach a maximum decreases as the concentration is raised.

The value of the maximum rate of heat production is shown in Figs. 1 and 2 as increasing rapidly with increasing strengths of K up to 9 times normal and at a slower rate thereafter up to 22 times the K concentration

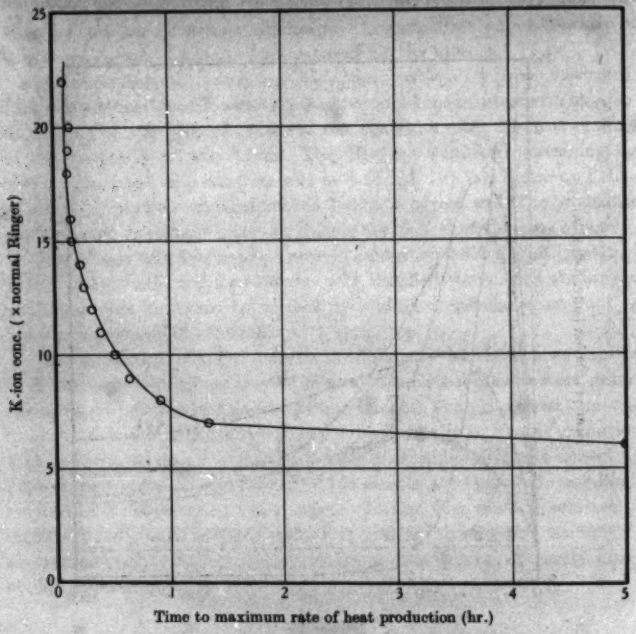

Fig. 2.

of normal Ringer's solution. In concentrations of over 22 times normal, the maximum had already been attained before a reading could be made.

Although there is this general correlation between the maximum level of heat production and the strength of K used, the individual results vary so much that it is evident that some other factor, uncontrolled in these experiments, must be operating. In preparing the curves of Fig. 1, the observed values were adjusted to a common initial average rate of resting heat production.

It is of interest that the time course of the rise and fall of metabolism was not subject to the variation shown by the initial levels of resting heat production.

In addition to the liberation of heat shown in Fig. 1, it was found that there was a brief explosive outburst of heat occurring immediately the K solution was added. This was first recognized at the higher concentrations when it was noticed that during the 2 min. before taking the first reading the galvanometer beam first fell from a high value and then rose to reach the value plotted in the curves of Fig. 1. On investigating further it was discovered that though decreasing in size with decreasing concentration, this initial liberation of heat could still be recognized at 8 times the normal concentration of K. The amount of heat liberated in this early rise increased as the concentration of K was raised up to the point where it could no longer be separated from the secondary delayed increase.

To determine if the secondary rise was the direct result of the early brief increase the KCl solution was replaced after the first 5 min. by the original Ringer which had been kept in a separate container in the bath, and now the metabolism returned directly to the original level within 10 min. Evidently, then, the secondary rise is dependent on the continued action of the high concentration of K.

The average resting value of heat production was 5·91 millicalories per g. per min. at 29·4° C. This varied from 3·75 to 9·20 in a series of 14 determinations.

The mechanical changes in the muscle were observed as described. In Ringer solution the isolated muscle occasionally showed fibrillar twitchings, 1 to 3 per min., throughout the first hour it was observed. With concentrations of K up to 4 times that in Ringer's solution no increase in the amplitude or frequency of the twitches occurred. Where twitching had been present in Ringer's solution it soon ceased under the influence of the increased strength of K. Beginning at a concentration of 5 times normal it was found that a brief series of twitches occurred when the solution was added. When these disappeared no further change was observed during the remainder of the hour. The twitches became more frequent and more numerous as higher concentrations of K were used. In all cases up to concentrations of 60 times normal all twitches ended within 4 min. The original length of the muscle was regained with the lesser concentrations, 15 to 20 times normal, immediately all the twitches ceased, and no shortening occurred in the succeeding hour. With higher concentrations there was a variable amount of residual shortening which passed off in 5 to 40 min.

Discussion

The above results indicate that K in excess of 4 times that of normal Ringer leads to two periods of increased metabolism. These are an immediate increase which in every case attains a maximum and falls rapidly towards zero within the first 4 min., and a delayed increase which has the variable time course shown in Fig. 1. Correlated in time with the initial phase of increased heat production are the twitches induced in the muscle by the stimulating action of the K, and they would appear to account for this increase in metabolism. No mechanical change could be observed in the muscle, however, to account for the delayed rise so that this is apparently a result of a stimulation of resting metabolism. As the latter was shown to depend on the continued action of K-rich Ringer's solution it cannot be simply an evidence of the recovery heat due to the initial twitches.

Hegnauer *et al.* [1934] showed that the oxygen consumption of the frog's muscle was increased by treating it with K, and that during the first hour the maximal increase was produced by a concentration of 100 mg. p.c., i.e. by 7 times the amount in Ringer. If the amount of heat liberated during the first hour is determined by measuring the areas under the curves of Fig. 1, we obtain results which may be considered as comparable, i.e. for the first hour the maximum increase is produced by 10 times the concentration of K in normal Ringer's solution.

The shape of the curves of Fig. 1 explains why Solandt [1936] failed to demonstrate any increment in resting heat production with high K concentrations. The technique used at that time would not detect a change in heat production as ephemeral as is the increment in resting heat production due to these high K concentrations.

Comparing the present work with the curves obtained by Fenn [1931], the changes in heat production may be considered as tending to parallel the changes in oxygen consumption when the muscle is treated with increasing amounts of K. Since Hegnauer *et al* [1934] were able to demonstrate a direct relationship between the oxygen utilized and the changes in phosphocreatine content of the muscle, it is probable that the increased heat production, like the increased oxygen utilization, is related to the breakdown of phosphocreatine and that a decrease in heat production similarly is related to a resynthesis of this compound.

The demonstration of the two phases of increased metabolism induced by K and their tendency to occur simultaneously in higher concentration may explain why chemical stimulation of muscle using isotonic potassium

sulphate was found to be associated with a higher oxygen consumption [Fenn, 1931] than electrical stimulation. The oxygen consumption for a given tension induced by K stimulation would be made up of the increase due to the shortening, and the increase in the so-called resting metabolism.

Summary

The increased heat production and the mechanical changes occurring in the frog's sartorius muscle when treated with potassium in concentrations from 1 to 60 times that of normal Ringer were studied. Two phases of increased metabolism were demonstrated, one associated in time with the twitching which occurred during the first 4 min. after immersion, the other appearing after this while the muscle is apparently inactive. The amount of heat liberated in the first 4 min. increases with the increase in the number and strength of the twitches induced as the K concentration is raised above four times the normal. The rate at which the so-called resting metabolism rises to a maximum and subsequently falls becomes progressively more rapid and the level attained is increased as the concentration is raised from 4 to 60 times that of Ringer.

It is with real pleasure that the authors acknowledge their indebtedness to Prof. C. H. Best for his invaluable encouragement and co-operation in this work. The technical assistance of Mr F. L. Robinson and Mr J. D. Brown has been of the greatest help throughout.

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J. Physiol. (1938) 93, 312-334 612.014.45:612.813

SYNCHRONIZED IMPULSE DISCHARGES FROM RECEPTORS IN THE DEEP TISSUES IN RESPONSE TO A VIBRATING STIMULUS

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(*Received* 11 *May* 1938)

In the present paper a series of experiments is described dealing with the effect of mechanical vibrations on the receptors in the deep tissues of animals.

It has been found that when a vibrating tuning-fork is applied to the bones or tendons of an animal, rhythmical discharges of impulses synchronized to the stimulation frequency can be recorded from the nerves of the neighbouring muscles. These discharges come from the "stretch" receptors.[1]

With this technique it has been possible to investigate the "stretch" receptors' response to stimulation at different frequencies. The response at high frequencies appears similar to that obtained in the auditory nerve and its mode of production seems analogous (after Wever & Bray's [1930] hypothesis).

In a brief preliminary note it was suggested [Echlin & Fessard, 1937] that the synchronized discharges described might be concerned in vibratory sensibility. This is of course a purely theoretical supposition, but is considered in detail in the final discussion, where the possible effect of the pattern of peripheral afferent discharges on central interpretation is mentioned.

General remarks on the problem of vibratory sensibility

Although the present paper deals with peripheral afferent discharges that may or may not give rise to any sensation, we are forced to speak of vibratory sensibility if we are to discuss the work of most past authors who have studied the effect of vibrations on sensory receptors: for before the advent of modern electrical methods sensation was an unavoidable intermediary and usually the only sign of peripheral activity in sense organs. It is not our

[1] "Stretch" receptors—those receptors in muscle and tendon which respond to stretch. See Matthews [1931 *a*].

intention, however, to give a detailed account of the literature on vibratory sensibility, as this is most extensive and in many cases too vague to allow of concise analysis; but rather to record briefly some of the views relating to its origin and to draw what conclusions we feel to be justified.

Any one who is acquainted with the literature on vibratory sensibility will be aware of the wide divergence of opinions as to the receptors responsible for its appreciation.

Some of the earlier workers [Rumpf, 1899; Weber, 1846; Treitel, 1896–7] were of the opinion that skin receptors probably are the most important in vibratory sensibility, but their evidence is entirely inadequate. Later von Frey [1915] and D. Katz [1923] gave definite evidence that the skin receptors are stimulated by vibrations, and came to the conclusion that they only are concerned in the appreciation of vibrations. They differ, however, as to the type of end organ involved, Katz believing it to be a specialized one and von Frey a pressure receptor. Adrian, Cattell & Hoagland [1931] have shown that single cutaneous receptors in the frog will respond at the same rate as a vibrating stimulus.

In opposition to the above beliefs, Head [1920], Déjerine [1914], Egger [1908], Neutra [1905], Piéron [1919], Symns [1917], Frank [1921], Bing [1905], Gordon [1936], Pollock [1937] and many others (which includes most neurologists) concluded that deep receptors certainly take part in the appreciation of the vibrations of the base of a tuning-fork. Most of the latter group of experimenters have felt that this method of stimulation affects largely the deep receptors, but some admit that those in the skin also participate. Many of them disagree, however, as to which deep end organs are responsible for the sensation.

Piéron [1919] feels that vibratory sensibility is evoked from any receptor either superficial or deep, and Bonnier [1898] likewise inclines to this view. Goldscheider [1904], although he admits the importance of deep sensibility, favours the cutaneous origin.

Head was firm in his opinion that only those receptors concerned in the appreciation of position and passive movements were involved, and remarks "the tuning-fork produces rapidly repeated movements of small range; these can be recognized, provided the appreciation of passive movements is shown to be normal". Egger and Déjerine, however, were equally convinced that receptors in the periosteum and possibly in the immediately neighbouring attachments (but not in the muscles) were peculiarly sensitive to vibrations. The evidence for this belief is supported mainly by the clinical observation that a tuning-fork produces its maximum effect when its base is placed upon a bone or joint, and little, if any, sensation of vibration results when it is applied to the belly of a muscle. This evidence, however, is not sufficient to rule out the possibility that the tendon and muscle receptors may respond; for most authors on the subject have pointed out that the bone is a particularly good medium for the transmission of vibrations. Neutra [1905] has suggested that these vibrations are reflected into the muscles which, he believes, contain the receptors for vibration, and Minor [1904] and Symns [1917] found that the response to a tuning-fork was unimpaired over bones that had been grossly affected by fractures or disease.

Apparently the only clinical objection to Neutra's suggestion is Egger's and Symns's observation that in certain cases of severe muscular atrophy, vibration sense was found to be normal. In fact, Egger showed that in some cases of unilateral atrophy, vibratory sensibility lasted longer in the atrophic than in the normal limb, "proof that the muscle is not the site of the perception of vibration". This objection can be disposed of, as Sherrington [1894] found that the muscle spindles ("stretch" receptors) are not affected by the muscular atrophy following section of the peripheral motor neurons, and by Batten [1897] and others who have shown that the muscle spindles are normal in cases of extreme muscular atrophy in man (i.e. acute poliomyelitis, progressive muscular atrophy, myopathies). Sherrington estimates that in the muscles of the limbs from one-half to one-third of the fibres in the muscular nerve branches are sensory and that most of these sensory

fibres end in the muscle spindles. These fibres, as is well known, in part end in the cerebellum and in part pass forward by way of the median fillet to end in the cerebrum.

It would appear, therefore, that there is no clinical or experimental proof to eliminate the possibility that receptors in muscles and tendons participate in vibratory sensibility, and this appears more than likely from Head's conclusion that "Thus from a developmental point of view vibrations and changes in posture are closely associated together and I believe that even in man they evoke the same physiological consequences." This possibility is also strengthened by Cohen & Lindley's [1936] observation that with increased muscle tonus there is a rise in the threshold to vibrations.

There is, however, a theoretical objection to the participation of these end organs, connected with the central effect of peripheral afferent discharges in response to a vibrating stimulus. This is: How could the discharges from a slowly adapting receptor, such as the "stretch" receptor (should they produce any conscious effect at all), give rise to other than a perfectly continuous sensation? We know that variations in frequency of afferent discharges from different receptors are interpreted centrally as changes of intensity. But we also know that variations in frequency can be recognized as such when a vibrating stimulus is used. Possible mechanisms that might be involved in this central discrimination have been theoretically considered and discussed by Wever & Bray for audition, Adrian [1935], Piéron [1935] and others for cutaneous vibration sense, and by us in the final discussion for deep vibratory sensibility.

Technique

In the present investigation we have used cats, rabbits and frogs. The mammals were anæsthetized with dial intraperitoneally and then placed in a steam heated box with a glass front, where the dissection was carried out. One of the animal's legs was fixed by means of a clamp about the foot and a nail driven through the suprapatellar ligament. Various nerves in a hindlimb were exposed and placed upon the electrodes which led to a condenser coupled amplifier. The nerve impulse discharges were recorded by a Dubois' oscillograph. The presence of a loud-speaker in the output circuit was of considerable value in distinguishing when synchronization of the impulse discharges occurred.

The same recording apparatus was used in the experiments with frogs. The preparations (the whole limb, the gastrocnemius muscle, or toe-muscle preparations) were fixed by pins driven through the bones in a small ebonite Ringer-containing chamber, the nerve extending on to electrodes in a separate compartment. The whole arrangement was similar to that used by Matthews [1931*a*]. Records were taken from various nerves in the hindlimb. For a single unit preparation, that described by Matthews [1931*a*] in the toe muscle was employed.

Tuning-forks of various sizes and frequencies (85–530 vibrations per sec.) were used for stimulation throughout. To insulate these, rubber was placed around the shaft and the base painted with shellac. Owing to this precaution, mechanical artefact rarely occurred, but when it did, was easily recognized by the sinusoidal type of wave which it produced in the

records. This type of stimulation has disadvantages; at high frequencies the amplitude of vibration is extremely small and rapidly damped. In cats we have not obtained an impulse response to frequencies above 530 per sec., and are unable to tell whether this is due to the low amplitude of vibration or to the fact that the receptors or nerve fibres are incapable of producing discharges above this rate. We have attempted to use various types of electrically driven vibrators, but so far have been largely unsuccessful either on account of electrical interference or due to the inadequacy of the type used.

Preliminary experiments

In order to determine whether receptors in the deep tissues of animals will respond to intermittent stimulation, we applied vibrating tuning-forks in different ways to the lower limbs of cats, while recording from the sciatic, femoral, or common peroneal nerves. The skin was at first left intact below the knee and the base of the tuning-forks of 100–440 vibrations per sec. applied to all portions of the limb. We found that, on recording from the sciatic or common peroneal nerve, when the base of the tuning-fork was pressed against the tibia, the anterior surface of the ankle or on other bony points, a faint sound of the same pitch as the tuning-fork came from the loud-speaker. When using the peroneal nerve, if the animal's foot was plantar flexed so as to stretch the anterior tibial group of muscles, the sound increased in loudness but diminished usually or disappeared completely if the foot was dorsi flexed. This increase in loudness of the sound on stretching the tendons was especially marked when using a large fork of 100 vibrations per sec.

In many places where cutaneous innervation was intact, as demonstrated by light stroking of the hairs, application of the base of the smaller forks gave no synchronized response. However, if the vibrating prongs of the tuning-forks were brought in contact with these areas, very loud sounds of the same pitch as the fork were heard.

On crushing the nerve between the tissues and the electrodes, further stimulation failed to elicit a response, giving evidence that the sounds described were due to nerve impulses. Records of the discharges produced (Fig. 1A) showed complex waves of high amplitude which were regular in rhythm and synchronized to the same frequency as that of the tuning-forks employed.

After removing the skin from the cat's leg, the same type of discharge was obtained when stimulating either with the base (see Fig. 1B) or prongs of the forks, and similar increases in the responses were present

when the appropriate tendons were stretched. In the experiments where the base of the fork was used, the discharges appeared to be of equal loudness and amplitude both before and after the skin was removed, but in those in which we stimulated with the prongs somewhat greater responses seemed to be present with the skin intact. To avoid interference when employing the prongs of the tuning-fork, the cat's skin was kept dry, and insulating tape or dry cotton bound around the limb when the skin had been removed. The bases and shafts of the forks were of course insulated as described under "Technique".

Fig. 1. Cat. Synchronized sensory discharges recorded from the peroneal nerve. A, stimulation with the base of a tuning-fork (freq. 128) applied to the ankle. Skin intact. Time marker 1/5 sec. B, base of a tuning-fork (freq. 240) applied on a bone (tibia). Skin removed from the whole leg. Same time-scale.

The above observations showed that deep receptors are stimulated by the vibrations of a tuning-fork, and the fact that the responses increased during stretch of a tendon suggested that the "stretch" receptors might play an important role in the production of these discharges. Experiments were accordingly carried out to see if this were true.

Synchronized impulse discharges from the "stretch" receptors in muscles and tendons

Since the work of Adrian [1926], Adrian & Zotterman [1926], and Matthews [1931*a*, *b*, 1933], it is well known that if a tendon to a muscle be stretched there occurs in the nerve to the muscle a complex asynchronous discharge of nerve impulses as a result of the stimulation of many "stretch" receptors. In order to see whether these receptors would respond synchronously to a vibrating stimulus, a string was tied to the tendon of the gastrocnemius muscle in a frog and, while this was being

held under a fixed tension, the prongs of a vibrating tuning-fork were applied to it. Immediately in the loud-speaker the noise of the asynchronous discharge in the sciatic nerve, due to tension, was replaced by a loud sound of identical frequency to that of the tuning-fork.

A large number of similar experiments were done on cats and frogs with the same result. The records obtained when forks of 85–530 vibrations per sec. were used on cats (or 85–440 vibrations per sec. on frogs) show regular discharges of respective frequencies, but the height of the individual composite waves in the discharges varies (Fig. 2). This variance

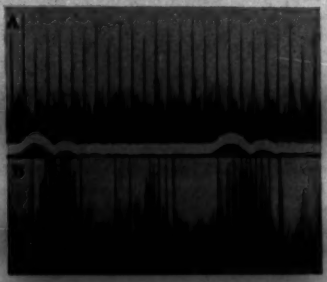

Fig. 2. Cat. Tibialis anticus muscle. Prongs of a tuning-fork applied on a string attached to tendon. The graphs show instances of responses A to low frequency (82 per sec.) and B to high frequency (530 per sec., near the upper limit) stimulations. Time marker 1/5 sec.

in wave height appears to depend mainly upon the amplitude of the vibration, provided the tension remains constant; for if a large fork is struck hard and immediately placed against the string, most of the asynchronous discharge disappears and the height of the individual waves in the synchronous one are of high amplitude (Fig. 3 B). When stimulating the frog gastrocnemius muscle in this manner, the asynchronous discharge is at times completely replaced by the synchronous one (Fig. 3 A). As the fork begins to vibrate with less amplitude, however, the waves in the rhythmic discharge become smaller (Fig. 4) and at the same time a more abundant background of asynchronous impulse discharge appears, if the steady tension applied is strong enough. Neither adaptation nor fatigue is responsible for the main features of this, for the experiment can be repeated over and over with the same result. Also when the same fork is struck very lightly or applied when its vibrations have become of low amplitude, the beginning of the resulting discharge appears like the end

of the other, that is, it is composed of a rhythmic series of waves of low amplitude superimposed on an asynchronous discharge. This same feature is evident in the discharges from high-frequency, low-amplitude forks. It

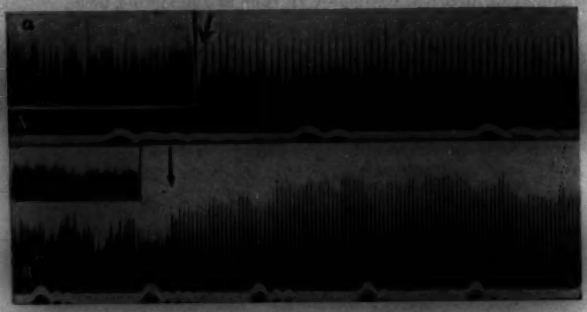

Fig. 3. Frog. Gastrocnemius muscle. A, B, asynchronous afferent discharge in response to a strong tension (100 g.) replaced from the arrow by a synchronized discharge when a tuning-fork was applied to the tendon. Time marker 1/5 sec. In B the left upper corner record shows irregularities of base-line at rest, and in A inset a shows the response to stretch—see text.

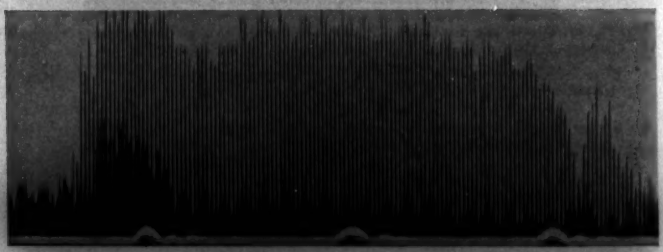

Fig. 4. Cat. Peroneus longus muscle. Synchronized impulse discharge from "stretch" receptors in response to stimulation with prongs of a tuning-fork applied to a string attached to the tendon. Frequency of stimulation 240. At the beginning, some receptors respond twice at each vibration. Time marker 1/4 sec.

follows of course from these findings that the duration of the discharge also depends upon the length of time the vibrations remain above a certain amplitude, and this is for only a fraction of a second with the highest frequencies, the fork being quickly dampened by the contact of the string.[1]

[1] Using an electrically driven vibrator, the frequency of which could be varied rapidly, it was possible to record a "tune" from the nerve.

Interpretation. That the discharges described above are due to the "stretch" receptors is evident from the fact that those resulting from constant stretch on the tendon largely disappear and are replaced by synchronous waves of high amplitude.

Since no evidence of synchronous discharge remains in some of the records from frogs' gastrocnemius muscles, it means also that all the "stretch" receptors in these particular cases are participating in the formation of the synchronous discharge, i.e. that all those which are stimulated by the stretch are capable of responding to a vibrating stimulus. The high amplitude of the individual waves in the discharges is, of course, the result of the summation of the individual impulses from many receptors. As fewer and fewer receptors respond synchronously when the amplitude of the fork vibrations diminishes toward the end of a single stimulation, the height of the individual waves accordingly decreases and the asynchronous background increases.

The discharges from "stretch" receptors in response to a vibrating stimulus applied to bone

Knowing that the "stretch" receptors are so sensitive to a vibrating stimulus, a number of experiments were performed to see if and how they would react when the stimulation was applied to a neighbouring bone. From the preliminary experiments we felt fairly sure that the "stretch" receptors would react, but the proof was still lacking.

After completely removing the skin from one leg of cats or rabbits, the experiments mentioned under "Preliminary experiments" were repeated in more detail and the same findings obtained.

The tendons were now divided about the ankle and a string attached to each. The strings were passed over a pulley, where they could be loaded with different weights and the impulse response to combined stretch and tuning-fork stimulation studied. With the base of a large tuning-fork of 100 vibrations per sec. on the tibia, little or no synchronized discharge appeared in the common peroneal nerve until one of the anterior tibial group of muscles was stretched. The best response could be obtained from stretch of the tibialis anticus when recording from the nerve to this muscle (Fig. 5). Even the slightest stretch (a few grams) on this muscle produced some synchronized discharge, at least at the beginning of the stretch, and this increased as more and more tension was used. The maximum synchronization occurred when between 50–100 g. were placed on the string. Weights greater than this also gave prolonged synchronized responses, but these did not always come on immediately.

The reason for this latent period will be discussed in some detail later. With the base of the tuning-fork on the upper insertion of the gastrocnemius the results were the same, synchronization of the response usually occurring only during stretch of the tendon.

In one cat, the peroneus longus muscle was completely removed from the animal, leaving the nerve attached to the muscle. Its upper insertion was fixed to a wax block and a string attached to the tendon. The whole preparation was kept warm by cotton soaked in warm Ringer, and before it died several experiments performed. It was found that on applying a vibrating tuning-fork to the string on the tendon, the usual synchronized

Fig. 5. Cat. Skin removed from leg. Base of a 100 D.V. tuning-fork applied on a bone. Sensory discharges during alternations of stretch and release of the tendon of tibialis anticus. A.C. main disturbances in the intervals. Time marker 1/5 sec.

discharge resulted. Also when the base of a large tuning-fork (100 vibrations per sec.) was placed on the wax, similar discharges appeared whenever the tendon was stretched. The results were the same when isolated preparations of the gastrocnemius muscle in the frog were used.

Discussion. The above experiments provide conclusive evidence that "stretch" receptors in the frog, cat or rabbit are stimulated by the vibrations of the base of a tuning-fork applied to bone and that, like bone, any solid object (wax block) under similar conditions can serve in the capacity of transmitting medium for vibration. Stimulation of apparently all the deep tissues of the leg gave no definite evidence that any other receptors respond to the vibration of the *base* of a tuning-fork, and if they do (i.e. if they take part in the meagre synchronized responses occurring without tension being applied to the tendons) we have found no evidence that the discharges from them are comparable to the massive response from the "stretch" receptors. We feel that the feeble synchronized discharges resulting from stimulation of the bone, when the neighbouring muscles are relaxed, may all come from "stretch" receptors closely applied to the bone, for it will be remembered in what intimate contact the tibialis anticus (and other muscles) are with the bones. Negative evidence, however, is not always trustworthy, and it is entirely

probable that other receptors in the deep tissues (such as the Pacinian corpuscles) will respond to a vibrating stimulus (if the amplitude be sufficiently large), and hence they may contribute in small part to the discharges described.

The responses from single "stretch" receptors

There are a number of problems of some interest which the above findings suggest, but little can be learnt concerning them unless single receptor preparations are used in their study.

The first problem is to determine whether the synchronized responses, which occur when a tendon is stretched during stimulation of the bone with a tuning-fork, are due mainly to the fact that the vibrations can more easily reach the receptors when the muscle is transformed into a rigid medium by the stretch, or whether other important factors are also involved.

For a single unit preparation, that in the toe muscle of a frog was used. It was set up in an ebonite chamber as described, tension being applied to the muscle through a string tied to the tendon. The vibrations were transmitted to the preparation by applying the base of a large tuning-fork of 100 vibrations per sec. to the side of the ebonite chamber. It was found that no impulses resulted on this type of stimulation until tension was put on the tendon. When the tension was gradually applied, no synchronized response appeared until it reached about 1–3 g. If it was now increased more or less rapidly so that the normal discharge from the receptor reached a considerably higher level than the rate of the vibrating fork, synchronization again disappeared. Below and above a certain frequency, therefore, the rate of discharge is determined by the stretch rather than by the vibrations of the fork.

The base of the vibrating fork (100 per sec.) was again placed on the ebonite chamber, and, 1 sec. after its application, weights of various sizes applied to the string on the tendon and photographs taken of the resulting discharges. As nearly as possible the fork was struck with the same force and the weight applied at approximately equal rates in each experiment. Naturally variables entered here, but they were partly eliminated by doing a large number of experiments. A mechanical apparatus for striking the fork with equal force each time was tried, but caused too much base-line fluctuations to be practical. Photographs were now taken of the normal response of the receptor to the above weights without any simultaneous stimulation from the tuning-fork. Five minute

intervals were allowed for recovery of the receptor between all experiments, and during these intervals the preparation was irrigated with fresh Ringer.

The results show that if the tendon be stretched by a weight of about 1–3 g., the receptor's discharge is not synchronized with that of the fork, except at the beginning of its response, when its rate of discharge approaches that of the fork, i.e. 100 per sec. With heavier weights (8–30 g.) the reverse effect is found, the discharge becoming synchronized to the rate of the fork only after a short interval. In this interval the receptor fires off at a higher rate than the tuning-fork. As adaptation to the stretch brings about a slowing in the rate, the frequency of the discharge suddenly becomes that of the fork, and for some time continues at this level (Fig. 6*a* and *c*), the duration depending mainly upon the amount of stretch and amplitude of the fork. After an interval, however, the impulse rate falls below that of the fork. Of course this latter effect is partly due to the decreasing amplitude of the fork vibrations; but if the fork is applied at varying intervals after stretch has commenced, it can be determined at what time, and at what frequency of discharge, synchronization no longer appears, or is replaced, as frequently observed, by synchronization at submultiple frequencies (Fig. 6*d*).

Discussion. The above experiments reveal that a "stretch" receptor is much more likely to respond at the same rate as a vibrating stimulus if, due to stretch, its own discharge rate approaches that of the stimulus frequency. In other words, if a receptor is already discharging at a rate close to that of a vibrating stimulus, it requires only a minute amount of vibration to synchronize its response to the vibration frequency. This behaviour can, therefore, partly explain the great sensitivity of the "stretch" receptor to a vibrating stimulus such as a high-frequency tuning-fork.

Another factor which apparently plays a role in the production of the synchronized discharges observed during stretch is probably that the stretch in transforming the muscle into a more rigid structure provides a better transmission medium for the vibrations. However, it has been shown that this is not the dominant mechanism, since in a muscle made quite rigid by the stretch of several hundred grams, no evident synchronization of the discharge occurs on stimulation with the base of a large 100 per sec. fork until a considerable interval has elapsed. During this interval the frequency of discharge from most receptors remains too high for synchronization to occur. Naturally, however, the length of this interval can be shortened by using vibrations of greater amplitude.

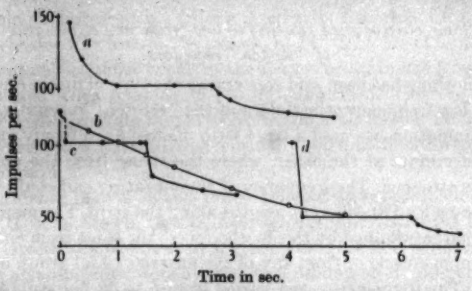

Fig. 6. Frog (single "stretch" receptor in toe muscle). Adaptation curves. In *a*, a steady tension 10 g., superimposed vibratory stimulation at 100 per sec. In *b*, steady tension 2 g., without vibratory stimulation; *c*, same preparation with tuning-fork applied; *d*, a little later, in response to a new application of the tuning-fork at full strength.

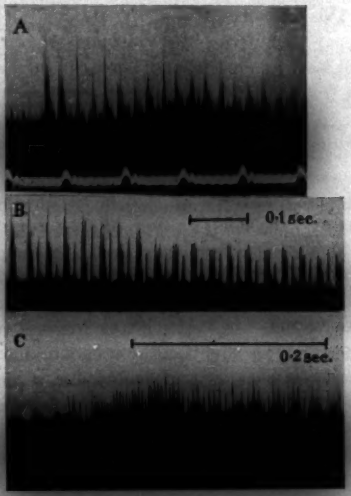

Fig. 7. A, Cat. Tibialis anticus muscle. Shows the impulse response of many receptors on stretching the tendon 25 times a sec. Recording from nerve to tibialis anticus. Time marker 1/5 sec. B, Frog. Toe muscle with several receptors. An instance of grouped impulses in response to a low frequency stimulation (32 per sec.). Average frequency in each group of three impulses: about 300 per sec. C. Shows the impulse response from a single stretch receptor in a frog on stimulation at 330 per sec. Time marker 1/5 sec.

Some observations relating to low-frequency stimulation of "stretch" receptors

So far nothing has been said concerning the type of discharge resulting from very low frequency stimulation of the "stretch" receptors. For this type of stimulation, we used a large strip of steel fixed firmly at one end, and free to vibrate at the other, where the string from the tendon of a muscle was attached. The frequency of the vibrating end could be varied by adjusting a load at different lengths along the strip. The main features of the discharge obtained by this method were the same from either frog's gastrocnemius muscle or from any of the anterior tibial group of muscles in the cat. The frequencies used varied from 5 to 32 per sec. As seen in Fig. 7 A the discharge is composed of a rhythmic series of composite wave potentials occurring at the stimulation frequency. The composite nature of each individual wave is much more evident than in the higher frequency discharges, where the waves are often formed by a single rather than a group of spikes. This difference is undoubtedly due to the fact that with the lower frequency stimulation the vibrations were of comparatively long duration and large amplitude, and hence caused a series of impulses to be discharged from the individual receptors at each stimulation (see Fig. 7 B).

Observations on high frequency stimulation of "stretch" receptors

Adrian *et al.* [1931] have shown that in response to an interrupted air jet directed against the skin of a frog, impulse discharges occur from single receptors at the same frequency as the stimulation rate. Even though the rate be raised to 250–300 per sec., the frog shows no sign of discomfort. This finding, as they point out, is extremely convincing evidence that the "touch" receptors in the frog cannot give rise to a sensation of pain no matter how they are stimulated.

We have found the same type of evidence in regard to the stretch receptors. Matthews [1933] suggested that high-frequency discharges from these receptors might give rise to a sensation of pain. Our evidence comes from the following experiments. If instead of tying a string to the tendon of a single unit toe-muscle preparation (frog), the tendon is left attached to the phalanges of the skinned foot and these allowed to hang in the air outside the ebonite chamber, a frequency in the nerve as high as 330 per sec. (at 22° C.) can be produced by bringing the prongs of a vibrating tuning-fork against the toe. This frequency is apparently close to the upper limit at which a "stretch" receptor can respond, and is

comparable to that resulting from a rapid strong stretch, i.e. 316 per sec. at 22° C. obtained by us and 290 per sec. at 14° C. by Matthews [1931*a*]. Similar frequencies must occur in the nerves of an intact frog if its toes are stimulated when hanging in the way described. There is no way in which the messages from these receptors could be further intensified, yet if this is done to a normal frog it usually pays little or no attention to the stimulation.

The evidence in cats on this problem is not as yet complete but seems definite. It is, as shown, that composite rhythmic discharges from many "stretch" receptors occurred at frequencies up to 530 per sec. when the prongs of a tuning-fork were applied to a tendon or at 440 per sec. when the base was placed on a bone. Frequencies over 240 per sec. have not been recorded from single receptors, but only two of these preparations have been studied, and in each case the receptor had a high threshold to stretch and was probably of the A_2 or B type described by Matthews [1933]. It is not surprising, therefore, that they were not stimulated by the feeble vibrations of a high-frequency fork. However, when one considers that a single muscle spindle in a frog will respond at 330 per sec., it seems hard to believe that there are not many "stretch" receptors in cats that will do so at 500 or over. Since the upper frequencies for stretch and for response to vibrations are so similar in the frog, it would appear more than likely that it is the same in the cat. In any case the cat shows no sign of pain if the base of a tuning-fork (or many tuning-forks) of any frequency is applied to a bony point or if the vibrating prongs are brought in contact with the tendons in its ankle or toes.

Discharges at submultiples of the stimulation frequency

The fact that the composite potential waves in the discharges in response to the higher frequencies of stimulation, like those in the eighth nerve, occur at the same frequency as the stimulation, suggests a comparison between the two phenomena. Adrian [1935] has already discussed a similar parallelism between the synchronized discharges in frog cutaneous nerves in response to intermittent stimulation and the impulses in the auditory nerve.

As is well known, the eighth nerve can transmit regular series of waves at frequencies of 1000 per sec. or over. It seems unlikely that a single fibre would conduct at this rate, and the current opinion [see Wever & Bray, 1930; Adrian, 1935] is that these high-frequency discharges, formed of composite potential waves, are probably the result of many fibres each conducting at a submultiple of the stimulus frequency.

Good evidence in support of this theory has been offered by Adrian, Bronk & Phillips [1931]; for they found that the eighth nerve was still capable of carrying these high frequencies, when the refractory period of the fibres had been increased to about three or four times its normal length, by either covering the petrous bone and auditory nerve, in cat, with crushed ice, or packing ice in the tympanic bulla of the guinea-pig. Evidence along the same lines has been found in the present investigation. It is as follows:

A. In the first place, as already mentioned, the highest frequency obtained from a single "stretch" receptor in a frog by intermittent stimulation was 330 per sec. at 22° C., which agrees fairly closely with Matthews's upper limit to stretch of 290 at 14° C. When working with frogs' muscles containing many receptors, however, frequencies of 440 per sec. at 17° C. have been obtained with ease on intermittent stimulation. Even after irrigation of these preparations with iced Ringer for a short time (30 sec.) frequencies of 440 have still been recorded, but on continuing the irrigation the receptors failed to respond to the fork.

B. Single "stretch" receptor preparations from frogs were set up as usual and stimulated by placing the base of a large vibrating tuning-fork (100 per sec.) on the walls of the ebonite chamber while maintaining a constant stretch on the tendon. As described above the records from this type of stimulation show that the discharge frequency becomes synchronized to the vibration rate when the response due to stretch approaches 100 per sec. When adaptation, due to maintained stretch, brings about a fall in the frequency, however, the discharge rate often suddenly becomes a half or a third that of the fork, i.e. 50 or 33⅓ per sec. (see Fig. 6*d*). Also if the preparation is subjected to stretch alone and the frequency of the discharge allowed to fall to 40 per sec., application of the fork (100 per sec.) will often cause a sudden rise of the rate to 50 per sec. From these experiments it would certainly seem that the receptor, under normal conditions, can respond at submultiples of the stimulation frequency.

C. Our final evidence comes from experiments on frog toe-muscle preparations containing two receptors. The experiments undoubtedly prove that "stretch" receptors will respond at submultiples of the stimulation frequency and that the composite discharge from a number of these receptors will, at the same time, reproduce the stimulation frequency through a process of alternation.

Preparations containing two receptors were selected. Just before stimulation with a vibrating tuning-fork (232 per sec.), a steady tension was applied to the muscle. In Fig. 8, part 1, an example is shown where

tension alone produced a discharge of 185 impulses per sec. from one receptor (*a*) and 165 per sec. from the other (*b*), the composite response being therefore completely irregular: then, as soon as the tuning-fork was allowed to exert its action (at *S*), each receptor took on the frequency of the stimulation and the complex discharge was abruptly replaced by one of double amplitude at 232 per sec. In part 2, after some adaptation to stretch had been allowed to occur ((*a*) 155 per sec., (*b*) 118 per sec.), the tuning-fork (232 per sec.) was again applied. In this case the result was a synchronized discharge at 116 per sec. From the height of the response it is obvious that each receptor was discharging at 116 per sec.

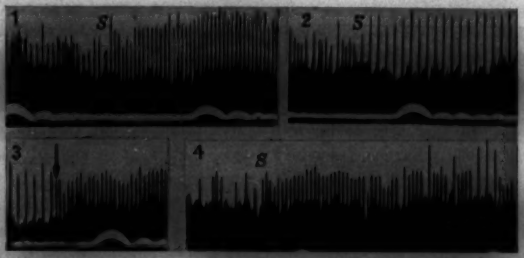

Fig. 8. Frog. Toe muscle under steady tension (10 g.) with superimposed vibratory stimulation (at *S*) during the course of adaptation. Two receptors. Example of responses at full (1) or half (2, 3, 4) frequency. Alternation can be seen in 3 (from the arrow) and 4 (from *S*). Time signal 1/5 sec.

In other records (parts 3, 4) it is seen that each receptor may be firing at half the stimulation rate but the total response be of the same frequency as the tuning-fork, each receptor responding to alternate vibrations. The amplitude of the waves in these cases is, of course, that of a single impulse.

Responses at submultiple rates are often preceded by transitional stages in which the response from one of the receptors keeps the normal rate while the other begins to show signs of deficiency. Such signs are of two kinds: first a progressive shift of the response from the second receptor, showing its tendency not to keep exactly in phase with that from the first and to be somewhat delayed with respect to it; and secondly, an occasional dropping out of an impulse from the second receptor's response. This may occur more and more frequently until every second impulse is dropped, i.e. the second receptor firing then only at half the stimulation

frequency. After each of these dropped impulses, the receptor may recover more completely and the shift mentioned above disappear, so that good synchronization of the discharge from the two receptors occurs. An analysis of a typical record is given in Fig. 9.

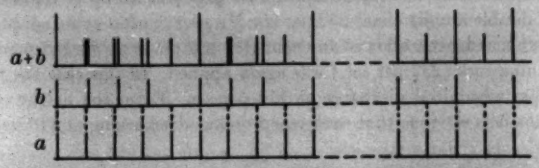

Fig. 9. Schema drawn from an actual record on 2 units. One of them (*a*) responds exactly in phase with the stimulation, the other (*b*) with some shift and failures until a rhythm at half frequency is obtained.

Discussion

Before entering into a theoretical discussion of the possible central effects of the discharges described in this paper, it seems advisable to review briefly some of the facts we have presented.

In the first place it has been shown that if a vibrating tuning-fork is applied in different ways to the limb of an animal, discharges from "stretch" receptors will result, and these will be synchronized to the rate of the stimulating vibrations.

Also, we have seen that, at least in most cases, the bones played a role of passive transmitters of these vibrations to the receptors in tendons and muscles, as originally suggested by Neutra [1905], and that vibrations had little effect upon the receptors if the muscles were flaccid. When some tension was applied to the tendons, however, discharges synchronized to the rate of the tuning-fork occurred in the corresponding nerves. The effect of the tension in these cases was apparently twofold. First by changing the muscle and its tendon into a more rigid structure a better transmission medium for the vibrations was provided. Secondly it was shown that the receptors became extremely sensitive to vibrations when they were placed in a state of activity by the stretch; for the nearer the discharge rate of a receptor approached that of the stimulating fork, the more likely was it to become synchronized to the vibration rate, i.e. the receptor's threshold to vibrations was lowered.

In the experiments, of course, this stretch (tension) had to be applied mechanically as the muscles were in a flaccid state, due either to the effect of the anæsthesia or to severance of nerves or tendons. In an intact

animal, however, the conditions would be quite different, for here, due to normal muscular tone, there would always be some stretch on the tendons and this would also be present on the muscles during extension. Matthews [1931*b*, 1933] has shown that in mammals many of the "stretch" receptors in tendon or muscle are active both during extension (stretch) and contraction. Therefore, in the normal animal, there would apparently always be many receptors discharging in any muscle (except perhaps during sudden relaxation or contraction of a muscle as in the "silent" period of reflexes), and hence in a condition to respond to vibrations. The impulse response to a vibrating stimulus would, however, vary with different tensions on a muscle.

So far in this paper nothing has been said of how the vibrations of a bone are transmitted to a muscle or tendon. It seems likely to us that the vibrations are not usually transmitted directly into the mass of a muscle, but stimulate many of the receptors, both in the muscles and tendons, by minute stretches of the tendinous attachments. These attachments are not only formed by the large tendons but are, of course, present along the whole length of most long bones, where they are in the form of thousands of tiny tendons.

The central problem

Peripheral aspects. So much for the facts. When one considers the possible effect of these discharges on the central nervous system, the ground is not quite so secure. It is known that postural tonus is a reflex from the sensory endings in muscle [Liddell & Sherrington, 1923]. When a vibrating tuning-fork is applied to a human bone, however, it evokes only a sensation of vibration, and usually no observable reflex effect on posture or tone. Yet, if our observations on animals can be applied to man, we must assume that it is causing massive discharges from the "stretch" receptors. The volume of these discharges will, as pointed out, vary with the state of the muscle tone, and this might explain why vibratory sensation fluctuates during contraction and relaxation of muscles.

Should these discharges produce no sensation it would mean that only deep receptors such as the Pacinian and Golgi Mazzoni corpuscles are concerned in the registering of the fine and complicated movements. This seems hardly likely, for they are apparently neither sufficiently numerous nor suitably placed to do so. It is more than likely that such receptors take a part in vibratory sensibility, but we have not been able to identify

their discharges and hence these must be small compared to the massive responses from "stretch" receptors.

As to the cutaneous receptors, the possibility that these play a role in vibratory sensibility, as explored clinically in man with the *base* of a tuning-fork, is not ruled out by our experiments on animals. In fact it seems probable that they do, especially in regions where the skin is more or less tense and richly innervated; for we know that they certainly respond to some kinds of vibratory stimuli [Adrian, Cattell & Hoagland, 1931; von Frey, 1915; Piéron, 1935, etc.], and it has been shown that they are stimulated by the vibrating prongs of a tuning-fork. However, true as this may be, they are apparently less sensitive to the vibrations of the *base* of a tuning-fork than the deep receptors, as Head has already suggested.

The possible effect of the pattern of peripheral afferent discharges on central interpretation

If discharges from "stretch" receptors give rise to any sensation whatsoever, and we take into account the present state of knowledge concerning central mechanisms, theoretical assumptions can be made which fairly support the view that the synchronized discharges under discussion are interpreted centrally as an intermittent stimulation.

To begin with, we need no assumption to say that, taken as a whole, the complex afferent discharges described offer special patterns that no other external stimulation than a vibratory one could produce, and as such might give rise to a specific sensation. But we can be less vague than this as soon as we admit that the ordinary fate of afferent series of impulses is to be smoothed out in the centres [see Adrian, 1935], so giving rise to a steady sensation; for a vibratory sensation may then be regarded as due to circumstances in which the smoothing effect is checked in some way resulting in large regular oscillations of the central excitatory state.

Adrian [1935] has pointed out that the discharge from a single slowly adapting receptor, such as the "stretch" receptor, cannot very well give rise to a sensation of vibration; for a constant stimulus to one of them will produce a rhythmical series of impulses (as it will at times from a touch receptor), the frequency of which is not interpreted as such, but rather as degrees of intensity, and any resulting sensation could only be continuous in nature.

However, at low-frequency stimulation it has been shown that, as expected, the response to vibrations of each single "stretch" receptor

often occurs in small groups of a few impulses separated by intervals of rest. Similar results have been obtained by Cattell & Hoagland [1931] working with skin receptors stimulated by intermittent puffs of air. It is quite obvious that such a response will result in regular fluctuations of the central excitatory state in each of the corresponding centres. Very distinct intervals of rest between groups are not necessary, and any uneven distribution of impulses, provided the fluctuations occur with regularity, certainly produces periodical variations in centres that may be interpreted as rapid modulations in intensity, i.e. vibrations.

All that has been said before concerns events that are supposed to occur in single receptors or centres. Such a simplification is rarely found in natural conditions. At any rate, even if a stimulation does occasionally affect a single cutaneous receptor, it seems hardly likely that this could ever be true of "stretch" receptors, for there are apparently always many of these end organs active in any muscle (except perhaps during sudden relaxation or twitch).

As is well known, the normal composite response to a steady stimulation of a group of either cutaneous or deep receptors is completely asynchronous. The sensation resulting, like the discharge, is a continuous one which rises and declines in intensity according to the number of receptors stimulated and the frequency of their individual discharges. When intermittent stimulation is applied to these receptors, however, a very different type of discharge is produced, at least from the "stretch" receptors (and "touch" receptors if the vibrations be strong enough); for here, as soon as the receptors feel the effects of the vibrations, the discharge from each one will become synchronized to the stimulation frequency (sometimes at submultiples of it) and the composite discharge will be a rhythmical copy of the stimulating vibrations. It seems hard to believe therefore that these discharges, so different from those resulting from steady stimulation, do not have a specific effect on the centres.

Synchronization of impulses as we have seen is not always quite perfect, and we must admit some spread during conduction toward the centres due to differences in velocities. These differences must be small, however, since only one type of impulse is concerned. In any case the impulses are undoubtedly distributed around a modal value, so that synchronization is relatively preserved (at least in the form of emerging crests in overlapping bursts of impulses) when the composite discharge reaches the centres. Obviously these periodical bursts of impulses counteract the smoothing effect if some central convergence is present. For then several impulses, meeting in the same place at approximately

the same time, would be capable of mutual reinforcement of their effects upon the centres.

Integration is a common feature of higher central mechanisms, and there seems to be no good reason why it should not occur in this case. Some experimental evidence of it being true, at least for the motor centres, is found in the observations made in man by Hoffmann [1919] and by Preisendorfer [1919]. These authors have shown that the normally asynchronous mass of impulses from the motor centres in a voluntary contraction can be transformed into large composite waves of the same frequency as that of the stimulus when strong vibrations are applied to tendons and muscles.

If the impulses in the discharges under discussion are integrated in their effect, a large fluctuation in central excitatory state should occur when each individual composite wave in the synchronized discharge arrives at the adequate centres. The separate fluctuations, if they give rise to any sensation whatsoever, could only, it would seem, be interpreted as fluctuations in tension or movements (or as contact in the case of the "touch" receptors), and deep vibratory sensibility would then be the appreciation of rapidly repeated minute passive movements.

This is just what Head has already visualized, for he says: "The tuning-fork produces rapidly repeated movements of small range" and "from the periphery to the cortex vibration and passive movements evoke sensation or are abolished together—in both of them sensation is evoked by afferent impulses caused by a series of minute passive movements."

By different methods then we have come to the same conclusions as Head. We wish it clearly understood, however, that our interpretation of the findings, as concerns vibratory sensibility, is a purely theoretical one, and it still remains possible that the discharges we have described never reach consciousness.

What pathways these high amplitude synchronized discharges follow in the C.N.S. and whether synchronization is maintained above the reflex level in the spinal cord could probably be determined by exploration of the C.N.S. with concentric needle electrodes and possibly by recording from the sensory cortex.

Summary

1. The vibrations of a tuning-fork will stimulate the "stretch" receptors in muscle and tendon if the base of the fork be applied to a neighbouring bone, or its prongs be brought in contact with a tendon.

2. In response to this type of stimulation the "stretch" receptors give rise to afferent discharges composed of rhythmic series of composite waves synchronized to the rate of the vibrating stimulus.

3. The individual composite waves are the result of the summation of impulses from many receptors. Within certain limits the number of receptors participating rises with an increase in the amplitude of the stimulating vibrations (tension remaining constant).

4. When the stimulus rate reaches a certain level, a "stretch" receptor produces a single impulse in response to each vibration, up to a certain high-frequency level.

5. The impulse discharge from a "stretch" receptor is much more likely to become synchronized to the stimulation rate if, due to stretch or contraction of the muscle, its rate already approaches the vibration frequency. Under these conditions its threshold to vibrations is lowered, and this explains in part the extreme sensitivity of "stretch" receptors to tuning-fork stimulation.

6. Bone serves as a transmitting medium for vibrations and causes many of the tendons fastened to it to be stretched sufficiently to stimulate the receptors in muscle and tendon at each vibration. By tendons we mean tendinous attachments which are present along the greater part of most long bones, as well as the larger tendons.

7. Evidence presented strongly suggests that discharges from "stretch" receptors are incapable of giving rise to a sensation of pain.

8. In response to intermittent stimulation, two "stretch" receptors may each respond at half the frequency of stimulation, yet the composite discharge be synchronized to the stimulation rate. Thus a proof that a high-frequency discharge may occur in a nerve when the separate fibres are carrying discharges only at submultiples of this frequency is offered.

9. It is suggested that the synchronized discharges from "stretch" receptors, produced by tuning-fork stimulation, might be largely responsible for deep vibratory sensibility. A theoretical discussion in support of this hypothesis is presented.

10. If this be true, then the same end organs are concerned in deep vibratory sensibility and the appreciation of changes in posture.

We take pleasure in expressing our thanks to Prof. Piéron for his many helpful suggestions and encouragement during the investigation and to Prof. Adrian for his criticism of the paper.

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J. Physiol. (1938) 93, 335-348 612.792.1:535.34

ON THE ABSORPTION OF ULTRA-VIOLET RADIATION BY HUMAN SWEAT

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(*Received* 18 *May* 1937)

This investigation was made in order to test an opinion commonly held that sunburn occurs less readily in man when he is sweating freely than when his skin is dry and free from sweat. Published results on the subject are somewhat contradictory [Coblentz & Stair, 1933, 1935; Hill & Eidenow, 1923–4; M. de Laroquette, 1920], and it therefore appeared to us that the results of our enquiry might be worth recording.

In this paper we offer experimental evidence in support of the view that human sweat, by virtue of its partial opacity to ultra-violet light, affords some very slight protection to the skin against those rays which are capable of producing erythema. Preliminary observations by one of us [Crew, 1935] have shown that a millimetre film of sweat absorbs slightly at a wave-length 3300 A., very appreciably at 2900 A., and almost completely at 2700 A. Our present experiments show that the interposition of a film of human sweat between a quartz mercury arc and the skin will definitely reduce the erythema normally produced. We shall describe these experiments and others by which the extinction coefficient of filtered sweat has been determined. Finally, we shall discuss the chemical composition of sweat and its bearing upon the absorption of ultra-violet radiation.

The fact that sweat collected from the skin surface contains not only the secretion of the sweat glands but also some secretion from the sebaceous glands has been recognized, and preliminary experiments have been undertaken to eliminate the effect of the suspended and dissolved fatty substances. The term *sweat* will be used to include these fatty substances, except when it is specifically stated that they have been removed.

Experimental

Most of the specimens of sweat used were obtained from a single subject "A", who is a man aged 36 years of normal health and physique. For occasional other specimens we are indebted to the members of the Cambridge University Gymnastic and Ju-Jitsu Club.

With subject "A" sweating was stimulated by running half a mile when very warmly dressed and sitting in front of a fire, or by taking a hot bath. The first sweat to appear on the forehead was wiped off and the remainder was caught in a clean test-tube. As much as 5 c.c. at a time could be obtained in this manner.

Screening experiments

In these rather crude qualitative experiments erythema was induced on the bare skin of one of us (C.H.W.) by means of the ultra-violet rays from a quartz mercury-vapour water-cooled Kromayer lamp filtered through various films of sweat, with the object of ascertaining (1) whether the sweat exerts any screening effect against the production of erythema; if so, (2) what thickness of sweat film causes a just detectable diminution of erythema; (3) whether the screening is due to the soluble substances or to suspended matter in the sweat; and (4) whether it is due to lipoid substances soluble in ether. These four points were examined in experiments A, B, C, and D. Experiment E was a control exposure through normal physiological saline and through tap water.

The procedure in these experiments was to direct upon the skin the total radiation from the lamp working under standard conditions. Between the lamp and the skin was placed a cell containing the sweat. This cell consisted essentially of two flat circular crystal quartz plates placed one above the other, each of 4·5 cm. diameter and 2 mm. thick. They were held in a thin brass flange by a spring clip and were separated by one of several iron wire rings varying from 0·2 to 1 mm. in thickness. The lower plate was held firmly in contact with the skin surface by manual pressure upon the brass flange. A drop of sweat was placed at the centre of the cell between the two plates, and when compressed to the thickness of the wire ring spacer used it presented a film about 1 sq. cm. in area. The lens of the lamp was placed in direct contact with the upper quartz plate when an exposure was made. In this way a disk of skin the size of the plates was exposed to rays passing through quartz and air, in the centre of which was an area covered by the sweat film. Erythema produced in this way appeared as in Fig. 1*a*. When the 1·0 mm. spacer

was used the thicker film of fluid tended to shift to one side of the cell so that the resulting erythema was as shown in Fig. 1*b*.

The results of these experiments indicate that sweat does provide some protection against the production of erythema, that a film of sweat 0·2 mm. thick gives a just detectable but definite screening effect, and that this effect is only partially due to suspended matter.

Fig. 1. *a*: Illustrating the appearance of erythema on the skin. The pale central area is due to the screening effect of a film of sweat in the centre of the cell. *b*: Illustrating the appearance of erythema on the skin. The unpigmented area towards the right is due to the screening effect of sweat directly above it in the cell. *c*: Illustrating markings made on skin. The right half of the circle was painted with an ether extract of sweat.

Exp. A. Exposures were made on the inner surface of the forearm or the thigh and the 1·0 mm. spacer separated the plates of the cell. A test exposure of 1·5 min. was made with no sweat in the cell. This produced, after a lapse of 8 hr., a uniform patch of erythema; and after 14 days marked pigmentation and scaling.

With sweat in the cell and the same exposure time a patch appeared somewhat as in Fig. 1*b*, showing only a trace of erythema under the sweat with a ring around this of marked erythema. After 14 days the effect under the sweat was nil, but the rest of the exposed area showed pigmentation and scaling.

It is clear that sweat in a film of this thickness, 1 mm., is an effective screen against the erythema- and pigment-producing rays from a mercury vapour lamp.

Exp. B. Exposures were made as above, but the sweat film used was only 0·2 mm. thick.

It was found that an exposure of 1¼ min. produced no erythema; that an exposure of 2 min. gave uniform erythema, with later pigmentation and scaling; and that 1½ and 1¾ min. exposures gave rise to an erythema which was quite obviously less intense in the sweat-screened area than in the unscreened surrounding zone.

If the skin be considered as a high contrast photographic plate and erythema expressed quantitatively and plotted as a function of the

intensity of incident radiation the curve would be a steep one. Since the intensities of radiation required to produce a definite erythema bear an inverse ratio to the corresponding times of exposure [Coblentz, 1931], and since it took 1¾ min. to produce the same intensity erythema through sweat as against 1½ min. without the sweat screen, the 0·2 mm. film of sweat appears to transmit 1½ : 1¾ of the incident radiation, i.e. 86 p.c. approx. This rough estimate agrees with the value, 82 p.c., obtained by spectrophotometric experiments to be described below.

Since the differential effect obtained is slight with a film 0·2 mm. thick, it is unlikely that thinner films would give perceptible diminution in the erythema.

Exp. C. In this experiment sweat which had been filtered through ordinary filter paper was used in the cell in a film 0·2 mm. thick. Exposure at 1¾ min. again showed a differentiation between the sweat-covered area and the surrounding zone.

Similarly, sweat passed through a Chamberland L 2 filter candle was used, this time in a film of thickness 0·6 mm. At 1½ min. exposure, an erythema was produced which was less intense in the central sweat-screened area than in the surrounding zone.

It appears therefore that filtered sweat retains some of its power of absorbing ultra-violet rays which produce erythema, and that dissolved substances are at least partly responsible. The relative powers of absorption for filtered and unfiltered sweat have been determined in spectrophotometric experiments to be described later.

Exp. D. This experiment was designed to give a rough idea as to the importance of possible lipoid substances in the screening effects observed. It is known [e.g. MacKenna, 1937] that sweat contains traces of fat, fatty acids and cholesterol. It is probable that a portion at least of these substances comes from the sebaceous glands and mixes with the secretion of the sweat glands. If such lipoids are present in suspension one would expect that simple filtration of the sweat through filter paper, or through a filter candle as in the last experiment, would remove them. The filtrate was, however, found to retain some of its screening effect.

The question whether this was due to dissolved lipoid substances was investigated by trying to remove any dissolved lipoids present by ether extraction. 1 c.c. of sweat was repeatedly shaken up with small quantities of ether, and the ether separated, until 15 c.c. of ether had been used in extraction.

The ether fraction was allowed to evaporate down to 1 c.c. and this

was painted on a small area of skin six times and allowed to dry. The painted area was irradiated, together with unpainted areas of skin, and the results as shown in Fig. 1*c* were compared. It was found that when 2½ and 3 min. exposures were given, an erythema followed which was slightly less intense in the ether-painted areas than in the unpainted, suggesting that the ether fraction did contain substances which absorb erythema-producing rays.

The residue after ether extraction tested again in the quartz cell was still found to retain some of its absorptive power. Since only the simplest method of extraction was used, the extraction could not have been complete, and this presumably explains the residual absorption.

Exp. E. The aim of this experiment was to make certain that neither water nor physiological saline exerted any screening effect in the sweat. Tap water and 0·8 p.c. sodium chloride in distilled water were employed respectively in the cell, and it was found that in neither case was there any evidence of absorption of the erythema-producing rays. (Kouno [1934] gives the concentration of sodium chloride in sweat as varying from 0·18 to 0·95 p.c., according to different workers.)

Absorption experiments

The absorption of light by human sweat was examined spectroscopically by illuminating the slit of a spectrograph with the continuous radiation from a hydrogen discharge tube and interposing films of sweat of different thicknesses. It was observed that for sweat films of 0·1, 0·5 and 1·0 mm. thickness, absorption of radiation began at about 2500, 3000 and 3500 A. respectively, and increased toward shorter wave-lengths.

The extinction coefficient of sweat in the region between 3500 and 2500 A. was determined by means of a Hilger spectrophotometer of the "Spekker" type [Twyman, 1931–2, 1933].

The results of this series of experiments are given in Table I; the first column represents the wave-lengths of radiation expressed in Angstrom units, and the second and third columns the corresponding values of the log extinction coefficients, computed from the relation: $J = J_0 10^{-Kl}$ (see *International Critical Tables*, **5**, 359), for unfiltered sweat and for sweat filtered through ordinary filter paper, respectively.

The values of log K shown in Table I were obtained by taking the logarithm of the mean of the values of K computed from seven independent experiments. The thickness, l, of the specimens used varied from 0·5 to 5·0 mm. From the conditions of the experiments, the values

of K are most accurate in the region 3000 A., where the probable error of the mean is about 2·5 p.c.

TABLE I

λ in A.U.	$\log_{10} K$ Unfiltered	Filtered
3500	0·61	0·12
3250	0·69	0·28
3000	0·77	0·53
2750	0·91	0·85
2500	1·04	0·91

DISCUSSION OF RESULTS

The protection which sweat affords against actual solar radiation may be estimated quantitatively from the absorption experiments. In this connexion we have plotted [Hausser & Gauer, 1933], as curve I of Fig. 2, the ultra-violet end of Petit & Nicholson's [1933] solar radiation curve, the ordinates representing radiant energy in watts per second falling on 1 sq. m. of surface. Curve II shows the relative erythema sensitivity of the human skin for uniform wave-length distribution of energy, according to Hausser & Vahle [1922]. The product of the corresponding ordinates of these two curves is given by curve III, which shows the relative importance of wave-lengths of sunlight in producing erythema. This curve shows that the most effective region of solar radiation, which we shall hereinafter call the "sunburn region", lies between 3110 and 2960 A. The maximum lies at about 3040 A. and not at the peak of the erythema sensitivity curve (2967 A.).

The transmissivities, J/J_0, for a 1 mm. film of sweat, have been calculated from the values of log K given in Table I, and are shown plotted as a function of wave-length in Fig. 3. From this curve it is seen that in the "sunburn region" only about 27 p.c. of the incident energy is transmitted by a 1 mm. film of sweat.

The loss of 73 p.c. of the energy in the transmitted beam in this experiment may be attributed to two causes; first, the pure molecular or colloidal absorption by the chemical constituents in solution and, secondly, the lateral scattering and reflexion of light by particles of skin, broken down cells, and other foreign matter suspended in the sweat. That a considerable part of the apparent absorption is due to scattering by foreign particles is evident from the results of measuring the absorption of filtered perspiration. The effect of filtering is shown in Fig. 3, where the transmissivity for a 1 mm. film of sweat is seen to increase from 30 to 56 p.c. at 3110 A. and from about 23 to 40 p.c. at 2960 A. Thus, even

filtered sweat, at 1 mm. thickness, transmits only about one-half of the incident radiant energy in the "sunburn region".

The actual thickness of sweat on the human skin is, of course, a decidedly variable quantity. It may be considered negligible for "in-

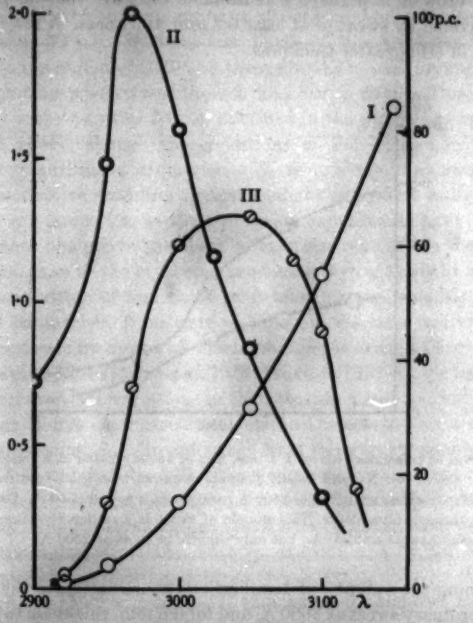

Fig. 2. I: Solar radiation curve [Petit & Nicholson], expressed in watts per square metre per second (left-hand ordinates). II: Curve showing sensitivity of human skin to the production of erythema for uniform distribution of radiant energy [Hausser & Vahle], expressed in percentages of maximal effect (right-hand ordinates). III: This curve is obtained by taking the products of the ordinates for curves I and II and multiplying by a constant, e.g. 2·5 (the left-hand ordinate numerals are used). It illustrates the relative importance of the wave-length of sunlight in producing erythema.

sensible sweating", and even for profuse sweating the liquid stands in droplets of various sizes and does not cover uniformly the whole surface of the perspiring skin.

The *average* thickness of the sweat on the forehead of one of us (W. H. C.), when profusely sweating, was found to be about 0·07 mm.

This figure was computed from the measured mass of sweat absorbed by a piece of blotting paper of known area, assuming the density of sweat to be unity.

From this result and from the appearance of the drops on a profusely sweating brow it is perhaps a reasonable estimate that 30 p.c. of this area is covered by sweat of at least 0·2 mm. thickness. A few drops were certainly of still greater thickness.

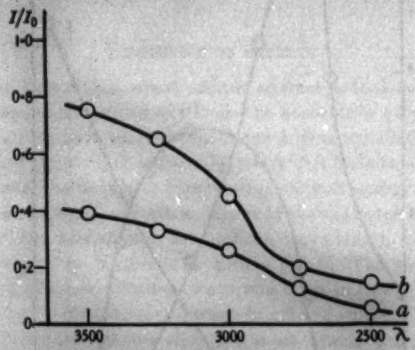

Fig. 3. Curves of transmissivity for a 1 mm. film of sweat plotted as a function of wavelength. Curve (*a*): Natural sweat. This curve shows that the sweat transmits only about 40 p.c. of the radiation at 3500 A., and about 6 p.c. at 2500 A. Curve *b*: Sweat filtered through filter paper. This sample of sweat is considerably more transparent than natural sweat at 3500 A., but only slightly more so at 2500 A.

Assuming the validity of Lambert's law, we calculate the transmissivity of ordinary sweat at 3000 A. and for 0·5 mm. thickness to be 71 p.c.; and at 0·2 mm. thickness, to be about 90 p.c.

Where there is scattered light Lambert's law does not hold accurately. It is probable, however, that approximately 30 p.c. of a sweating skin which is exposed to the light of the sun will receive only about three-quarters of the incident radiant energy from the sunburn region of the spectrum.

It is highly probable, however, that the actual protection afforded by sweat may be greater than suggested by the above figures in virtue of the fact that in prolonged sweating there will be an accumulation of natural greases and an increased concentration of dissolved substances due to evaporation of water from the excreted sweat.

In the screening experiments, previously described, erythema was induced by the total radiation from a quartz mercury arc lamp. In that case the strong mercury lines near 2540, as well as those near 3000 A., were effective [Forsythe, 1937; Duggar, 1936] in producing erythema. The transmissivity of a 0·2 mm. film of sweat in the region 2540 A., calculated from our experimental determination of the extinction coefficients, is 59 p.c. If about one-fourth of this erythema is due to radiation in the region of 2540 and three-fourths to that around 3000 A., the average transmissivity of the 0·2 mm. film is 82 p.c. This compares favourably with the value 86 p.c. obtained in the screening experiments mentioned above, though the conditions of the latter hardly justify any strict quantitative comparison. It is unlikely that sweat would provide as effective shielding against sunlight as against radiation from a mercury arc lamp; for, as the absorption experiments have indicated, sweat is more opaque to the short ultra-violet rays of the mercury arc around 2500 than to the erythema-producing rays of sunlight at 3000 A.

The composition of human sweat is known to vary widely from one individual to another, from time to time for the same individual, and with the methods by means of which sweating is excited [Kouno, 1934; McSwiney, 1934; Talbert *et al.* 1933; Mosher, 1932–3]. We have sought to find corresponding variations in the absorption spectra, but find none outside our limits of experimental error. The transmission curves, in the sunburn region, for filtered sweat from the various members of the Ju-Jitsu Club who had been exercising vigorously varied not more than a few per cent from similar curves for sweat from subject A, obtained by running. Likewise, numerous specimens of sweat from A taken at different times and stimulated both by exertion and by hot baths gave nearly identical transmission curves.

It is known [McSwiney, 1934] that sweat decomposes rapidly due to bacterial attack upon the urea, and we were interested, therefore, to see whether one could observe any variation of the absorption spectrum with time. At best we were not able to make a spectrophotometric observation of sweat within less than an hour after its excretion; but from 1 hr. to 2 weeks after its excretion there was no detectable change in the absorption of a specimen kept at about 60° F. Changes due to bacterial action may, however, already have taken place between the time of excretion and the time of the first photometric examination of a given specimen.

Discussion of chemical constituents

In an effort to discover what chemical constituents of sweat may cause the true molecular absorption, such as is observed in curve *b* of Fig. 3, we have considered the absorbing power of the many substances contained in sweat. It is not possible at present to come to any definite conclusion about this point beyond saying that the observed absorption curve of filtered sweat is probably a composite of the corresponding curves of several constituents. In this connexion the following points deserve consideration.

The composition of human sweat has been studied by numerous investigators [McSwiney, 1934; Talbert *et al.* 1933; Mosher, 1932–3; Barney, 1925; Pemberton *et al.* 1929; Hoelscher, 1899 and 1904; Riggs, 1911; Plaggemeyer & Marshall, 1914; Whitehouse, 1935]. Even so Peters [1935] has recently remarked that the composition of sweat and factors that control it remain quite obscure. We have taken the table of constituents given by Mosher [1932–3] as being adequately reliable for our purpose and have computed the transmissivity of the individual substances for amounts actually present in sweat and for 1 mm. thickness, taking the values of the molecular extinction coefficients from the *International Critical Tables*.

Uric acid nitrogen in sweat amounts, according to Mosher, to about 0·3 mg. per 100 c.c., and it has been shown by Smith [1928] to have a strong absorption maximum in the region of 3000 A. This amount of uric acid would have a transmissivity in the sunburn region of about 90 p.c. Urea and lactic acid are practically transparent in this region. Ammonia and sugar are transparent to wave-lengths longer than 2700 A. Creatinine and creatine are present in too small amounts to contribute appreciably to the absorption.

The amount of amino-acid nitrogen in sweat is reported to be about ten times that of uric acid nitrogen. Since many of the amino acids are highly absorbing in the ultra-violet it seems probable that there may be some present which contribute to the opacity of human sweat. As far as we are aware the amino acids in sweat have not been identified except for serin, which has been reported by Embden & Tachau [1910]. According to the data of Hartley [1887], serin would provide negligible absorption in the sunburn region. The amino acids tryptophane and tyrosine have been found by Ward [1923] to absorb in the neighbourhood of 3000 A., and Lucas [1931] attributes the opacity of human epidermis for wave-lengths below 3130 A. principally to the presence of these two

substances. A sample of filtered sweat was tested for tryptophane and tyrosine but neither was found to be present.

The absorption curve of cholesterol [Heilbron *et al.* 1927] bears a slight resemblance to the curves for sweat; but if the figures given by Gillam & Heilbron [1936] in their latest paper are accepted, the quantity of cholesterol necessary to produce the absorption recorded for sweat would be impossibly large in view of its low solubility. It seems therefore that cholesterol is not a considerable factor in the absorption.

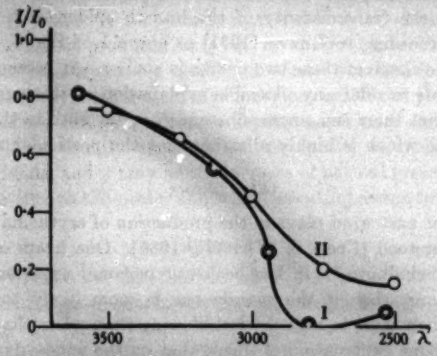

Fig. 4. I: Curve showing the transmissivity of human epidermis at 0·08 mm. thickness plotted as a function of wave-length [Lucas]. II: Curve showing transmissivity of human sweat at 1·0 mm. thickness [Crew & Whittle].

Eder [1935] reports that persons particularly susceptible to sunburn may increase their resistance to it by the introduction of iron into their diet. It appears from this, and other evidence, that the presence of iron in the skin reduces its sensitivity to erythema. This suggested the possibility that the iron in sweat produces a screening effect to ultra-violet light; the more so in that comparatively small amounts of ferric and ferrous oxides [Davidovich, 1930] and ferric chloride are highly absorbing at 3000 A. We found, indeed, that the shape of the transmissivity curve for ferric chloride was almost identical with that of filtered sweat. At our request the late Dr Hugh Ramage, of Norwich, very kindly tested a sample of sweat for the presence of iron by a method [Ramage, 1929] of spectroscopic analysis. The sample of sweat was from subject A, was unfiltered, and was reported by Dr Ramage as containing iron, but in amount not more than 0·0005 p.c. Probably much of this iron was

in the particles of skin and other foreign matter in the sample of sweat. This amount of iron, however, were it in solution as ferric chloride, would be almost completely transparent, at 1 mm. thickness, to radiation in the sunburn region.

With regard to other minerals in solution in sweat little can be said until more specific data are available in regard to their presence as chlorides and sulphates.

We have plotted together, as a matter of record, our curve (*b* of Fig. 3) for the transmissivity of a 1 mm. film of sweat as curve II of Fig. 4, and the transmissivity of the human epidermis for 0·08 mm. thickness, according to Lucas [1931] as curve I of Fig. 4. It is to be seen that the shape of these two curves is similar. At present, however, we are unable to offer any plausible explanation of this agreement. It is possible that there is a single substance present both in the epidermis and in sweat which is highly absorbent for the particular wave bands studied.

Other factors

Just what part wind plays in the production of erythema is none too clearly understood [Crew & Whittle, 1936]. One hears often of the complaint "windburn". It has been our personal experience that on a bright, sunny day at the seaside one is more likely to experience erythema when there is a good wind blowing than when the air is quiet and sultry. One explanation of this is that on the windy day one's skin is kept free of sweat and therefore of the partial protection against sunburn it otherwise would afford. An alternative explanation is that the wind itself produces erythema.

To test the point in hand we performed the following experiment. The inner forearm was covered with a piece of heavy rubber tubing from an automobile tyre, except for a small area of 1 sq. in. where the rubber had been cut away. In the absence of any ultra-violet light, the arm was then held for 30 min. directly in front of the "honey-comb" of an experimental wind tunnel generating a 40 m.p.h. wind. The relative humidity of the air was 50 p.c. and its temperature 66° F. During the exposure the skin exhibited "goose-flesh", but at no subsequent time was there any evidence of reddening or chapping of the exposed area of the skin. This result suggests that winds of less than gale force contribute little, if anything, towards directly causing erythema and pigmentation of the skin under the above conditions of humidity and exposure. The wind may, however, induce a secondary effect by rendering the skin more sensitive to sunburn. It would be worth while studying the reaction of

the skin to stronger winds at various temperatures, humidities, and times of exposure, both with and without the incidence of ultra-violet radiation.

At the beginning of this paper the question was proposed: Does human sweat actually screen the skin from the rays of sunlight which produce erythema? The foregoing observations quite definitely point to the answer that sweat does offer some slight protection. It seems probable, however, that a more influential factor is the physiological effect of sweat upon the skin in keeping it moist, as discussed by Hill & Eidenow [1923–4]. This point of view is supported by the wind-tunnel experiment in which we found that in the absence of ultra-violet light a very strong breeze produced, in ½ hr., no observable erythema. If the skin becomes sunburnt more readily in windy weather, it is probably due to a secondary, rather than a primary, action of the wind.

As far as we can judge from everyday experience activity in the sweat glands is usually accompanied by active secretion from the sebaceous glands, and it may be the increase of natural grease from these which is the clue to the protection which sweating seems to afford.

Summary

1. Experiments are described in which erythema is induced by the total radiation from a quartz mercury arc lamp. It is shown that the interposition of a film of human sweat partially screens the skin against erythema-producing radiation; that a 0·2 mm. film produces a just observable screening effect; that the screening is due to dissolved as well as suspended substances; and that probably the fatty substances soluble in ether are responsible for some of the screening effect.

2. A table listing the extinction coefficients of natural and filtered human sweat as a function of wave-length is presented. From this it is computed that a sweat film of 1 mm. thickness transmits only about 27 p.c. of solar radiation effective in producing sunburn.

3. The *average* thickness of sweat on the forehead of a person profusely perspiring has been found to be about 0·07 mm. and it is estimated that under these circumstances roughly 30 p.c. of the surface of the skin will receive only about 75 p.c. of effective solar radiation. Under prolonged sweating the protection against sunburn is probably somewhat greater.

4. The composition of sweat is discussed with regard to the absorption of ultra-violet light by its various constituents.

5. Exposure of the skin of the inner forearm to a 40 m.p.h. wind for ½ hr. produced no observable erythema or pigmentation.

For the use of the Hilger spectrophotometer and the numerous helpful suggestions in regard to its use we wish to record here our deep indebtedness to the late Prof. Lowry and to Dr Allsopp of the Department of Physical Chemistry at Cambridge University.

We should like also to thank Dr T. Moore of the Dunn Nutritional Laboratory, Cambridge, for his kindness in reading through the paper and for his criticisms and suggestions, and Dr W. S. Farren of the Engineering Laboratory for the use of a wind tunnel.

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J. Physiol. (1938) 93, 349-366 612.79:537.727

THE PHASE ANGLE OF NORMAL HUMAN SKIN

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(*Received* 18 *May* 1938)

Gildemeister [1928], Einthoven & Bijtel [1923] and Hozawa [1932] have measured the resistance and capacity of human skin in the audio-frequency range. The calculated phase angles show a decreasing

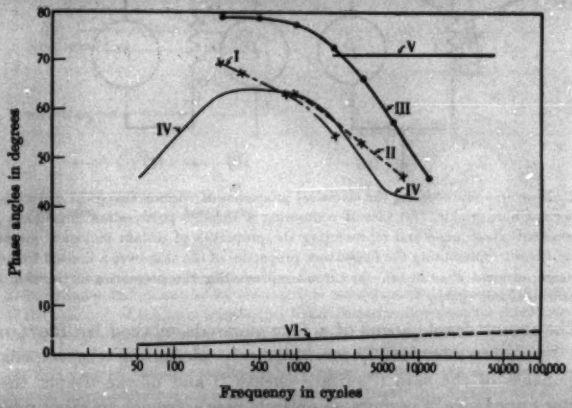

Fig. 1. Phase angle of the skin and deep tissues as a function of frequency according to various authors. I, Gildemeister; II, Einthoven & Bijtel; III, Hozawa; IV, Horton & van Ravenswaay; V, Present author; VI, Solid line, Horton & van Ravenswaay; dotted line, present author. Curves I–V refer to the skin, curve VI to the inner impedance (deep tissues).

characteristic with increasing frequency (Fig. 1). Cole [1932] has shown that, for biological materials as diverse as potato, frog nerve, rabbit muscle and frog skin, the impedance may be represented over a very wide frequency range by either one of two simple networks (Fig. 2*a*)

containing two resistances and a single variable impedance element of constant phase angle. From the existing data on human skin, this author [1933] has calculated the phase angle of the variable impedance element and finds that, whereas the measurements of Gildemeister and Einthoven & Bijtel give a value of about 55°, those of Hozawa run as high as 89–90°. Cole suggests that this wide divergence may be due to some systematic error in the bridge technique of the earlier authors. However, since precise experimental procedures for separately measuring the properties of the skin and of the underlying tissues were not available to any of these workers, it is not unlikely that the estimated effect of the deep tissues on the skin measurements may have been grossly at fault.

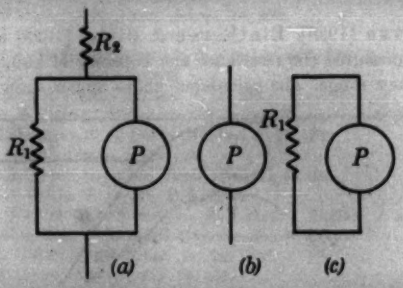

Fig. 2. Networks representing the electrical properties of various biological materials and normal human skin. (*a*) Circuit containing a variable polarization impedance P of constant phase angle and representing the properties of certain biological materials. (*b*) Circuit representing the impedance properties of the skin over a limited frequency range; element P as in (*a*). (*c*) Circuit representing the properties of the skin from zero to 40,000 cycles.

The recent development of a four electrode method by Horton & van Ravenswaay [1935] for separately measuring the impedance components of the skin ("surface sheath") and of the deeper tissues ("inner impedance") permits an entirely new approach to this problem. The experimental principle employed by these authors is the one embodied in the Kelvin double bridge [Hague, 1932] which is also a four terminal network. It will be recalled that this bridge was designed to avoid errors due to imperfect contact at the bridge arm terminals in measuring resistances of small value. This was accomplished by the simple stratagem of tapping off voltages across the terminals of the resistor under test and balancing these voltages, the effect of any contact resistance being thus eliminated. Although the original Kelvin double bridge was designed for

use with direct current, a number of A.C. bridges have since been developed embodying the same principle [Hague].

In applying the double bridge principle to body measurements, Horton & van Ravenswaay use a first pair of electrodes A–C (Fig. 3*a*) encircling the wrists or forearms as current terminals. The impedance

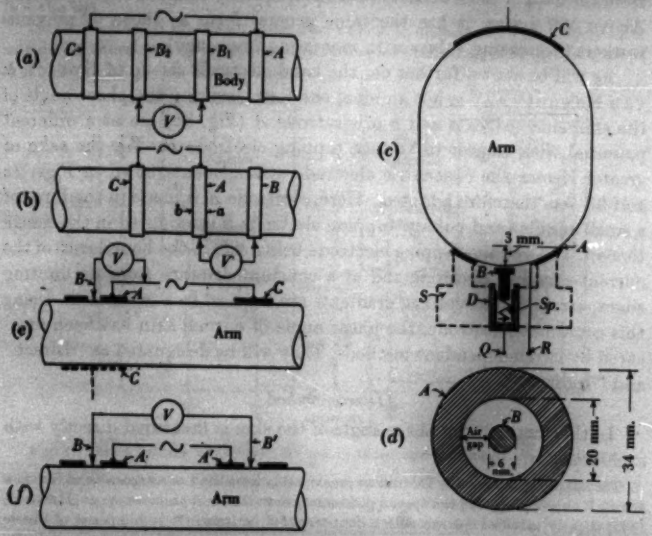

Fig. 3. Techniques and electrodes for the separate measurement of the electrical properties of the skin and of the deep tissues. (*a*) Band electrode technique for measurement of the properties of deep tissues through the skin. (*b*) Band electrode arrangement for skin measurements. (*c*) Details of electrodes for concentric electrode technique. (*d*) End view of concentric electrode. (*e*) Effect of varying the direction of current flow on measurements made with a concentric electrode. (*f*) Arrangement of concentric electrodes for separate measurement of the deep tissues.

properties of any portion of the body lying between these terminals such as the upper arm or chest may then be measured by tapping off voltages from a second pair of electrodes B_1–B_2 positioned to delimit the body segment being studied. Since the voltages tapped off in this way are independent of contact impedance, the effect of the skin is eliminated and the results obtained represent the properties of the deep tissues or "inner impedance" alone. If it be desired to study the properties of the

skin or "surface sheath", one voltage is tapped off at one of the current terminals such as *A* (Fig. 3*b*) and the other at some external point *B*. From reactance and resistance measurements made with an A.C. comparator either the *Q*-factor or the phase angle of the skin may be calculated. The curve shown in Fig. 1 represents the phase angle values calculated from the data of these authors over the frequency range 50–10,000 cycles. Above 500 cycles, it has the same general form as those of previous workers (decreasing values with increasing frequency).

As will be shown further on, the band electrode set-up of Horton & van Ravenswaay is not an ideal one for measuring the phase angle of the skin since edges *a* and *b* of electrode *A* (Fig. 3*b*) are at a different potential with respect to voltage tapping electrode *B*. For the sake of greater rigour, the concentric electrode arrangement shown in Figs. 3*c* and 3*d* was therefore adopted. Here, electrode *A* is made in the form of a small annulus and voltage-tapping electrode *B* is mounted in the centre thereof. The voltage-tapping electrode being within the boundaries of the current-carrying electrode and at a constant distance from its limiting edges, errors due to voltage gradients are reduced to a minimum. Using this concentric electrode, the phase angle of normal skin has been measured by two independent methods. They will be designated as "Direct" and "Indirect".

Direct method

In this method, the phase angle of the skin is measured directly with a phasemeter.

The measuring instrument. This differs considerably from the A.C. comparator of Horton & van Ravenswaay and is a special phasemeter described in a previous paper [Barnett, 1935] slightly modified for use with a four-terminal network. It is composed of phase-changing (Fig. 4) and phase-indicating units in all respects identical with those originally used and which need not be described again here. The number of terminals employed, however, is increased from two to three. Current is fed to the body between terminals 1 and 2 (ground) which are in series with a large resistance, and voltages are tapped off between terminals 3 and 2. The grounded current and voltage terminals being common, the four-terminal network is reduced in effect to a three-terminal system. Measurements were made at 15,300 c./sec. using a wattmeter as a null instrument and at 11,200 and 41,800 c./sec. with a cathode ray type of instrument. The phase angle indication in these phasemeters is read off directly from a phase changing dial *P* and remains independent of the impedance. Measurements made on impedances of known value showed the accuracy of the instrument to be within 1 p.c. By short-circuiting terminals 2 and 3, the phasemeter may be converted into the two-terminal instrument previously described.

The electrodes. These are shown in Figs. 3*c* and 3*d* and are of two types, concentric and simple. Concentric electrode *A–B* consists of (1) a monel metal annulus *A* covered with outing flannel and bent into cylindrical form (the curvature of the annulus is indicated in the drawing) to fit the upper arm and (2) a carbon voltage tapping electrode *B* measuring 6 mm. in diameter at the contact end and provided with a bronze spring *Sp* tending to force

the electrode into contact with the skin. Electrode *A* measures 34 × 20 × 1·0 mm. and has a total surface area of 6 cm.[2] Elements *A* and *B* are mounted together on a hard rubber support *S* and make separate connexion to terminals *Q* and *R*, all junctions being soldered including the one to the monel metal annulus. Electrode *B* is a brush holder taken from a Bodine Electric Co. Type *C*-3 motor and consists of a brass-lined hard rubber shell *D* fitted with a bronze spring *Sp*. Electrodes *A* and *B* should be mounted above the level of their support to leave an insulating air gap. This may be done conveniently by seating annulus *A* in a wax bed.

Simple electrode *C* is a monel metal disc, 34 mm. in diameter × 1·0 mm. also covered with outing flannel and mounted on an appropriate hard rubber support. It is immaterial

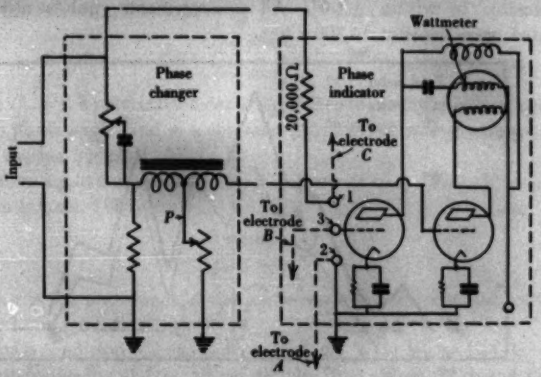

Fig. 4. Direct reading phasemeter for use in three- and four-electrode techniques.

whether this electrode be flat or bent, since perfect contact with the skin is unnecessary. The contact impedance of electrode *A* wetted with 1 p.c. saline and applied flat to a copper plate was found to be 0·5 ohm at 15,300 c./sec. and the phase angle of this system in series with a pure resistance of 100 ohms, 0·004 radian.[1]

Measuring technique. The skin is first gently wiped with acetone and permitted to dry and return to normal temperature. Electrodes *A–B* and *C* are then soaked in 1 p.c. saline and mounted by means of rubber bands on opposite sides of the upper arm (Fig. 3*c*), electrode *A–B* lying over the triceps and *C* over the biceps. Current is fed to electrodes *A* and *C* from the phasemeter and passes transversely through the arm, voltages being tapped off between electrodes *B* and *A*. Annulus *A*, it will be noted, serves as ground for both the current supply and voltage. With the electrodes in position, it suffices to rotate the phase changing dial of the phasemeter until the wattmeter or cathode ray tube indicates a null to

[1] The impedance of the skin area lying below annulus *A* is about of the order of this resistance in normal individuals. The phase angle value thus found represents the correction to be applied to the total phase reading taken as described further on.

obtain a phase reading. In making these readings, it will be observed that, during the first minute or two that the electrodes are in place, the phase angle gradually increases. This appears to be due to the diminution in contact resistance during the time that the saline is penetrating the epidermis. At the end of about a 2 min. period the phase angle becomes constant and repeated readings made thereafter give the same value. It is the final stable reading which is taken as representing the true phase angle of the skin. Phase angle determinations made in this way are reproducible to within $\pm 1{\cdot}0°$. All measurements, unless otherwise indicated, were made at 15,300 c./sec.

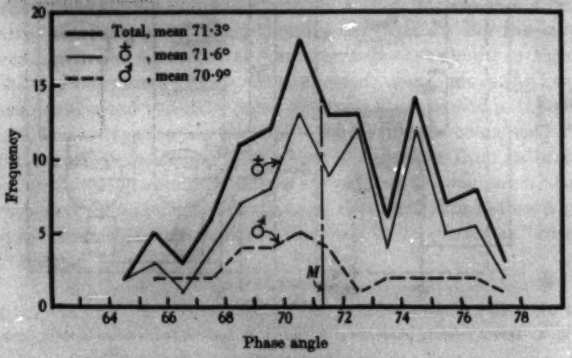

Fig. 5. Frequency polygon of normal phase angle values for 120 subjects; 87 females, 33 males.

Results. A number of initial readings made on two normal subjects showed values of 65° for one and 74° for the other. This appeared to indicate that the normal values extended over a considerable range and that, in order to find the limits of this range and determine its distribution, it would be necessary to make measurements on a relatively large number of individuals. Through the courtesy of Dr C. V. Bailey, 120 normal subjects (87 females and 33 males) undergoing a general physical examination were made available for study.

The results obtained are shown in Fig. 5. The phase angle of the skin was found to vary between 64° and 78° for different individuals with a mean value of 71·3° and a standard deviation of 2·85°. There is no significant difference between the distributions for males and females.

In eight unselected subjects (four males and four females) having an average phase angle of 72·5°, phasemeter readings were made at all three

frequencies available, i.e. at $11{\cdot}2 \times 10^3$, $15{\cdot}3 \times 10^3$ and $41{\cdot}8 \times 10^3$ c./sec. The results in all cases showed constancy with frequency to within $\pm 1°$.

Indirect method

Fricke [1932] has shown that, for a polarization impedance, if the polarization capacity varies as an inverse power function of the frequency, i.e. if the log (capacity)-log (frequency) curve is a straight line, the phase angle of the polarization impedance is constant and may be calculated from the following relations:

$$C_p = \text{const.} \times f^{-m}, \quad \text{.....(1)}$$

$$\psi = m.\pi/2, \quad \text{.....(2)}$$

where C_p is the polarization capacity, f the frequency, ψ the complement of the phase angle, and m a constant (slope of the straight line obtained from the log log plot).

From equations (1) and (2) it follows [Cole, 1934; Murdock & Zimmerman, 1936] that

$$z = \text{const.} \times f^{-\alpha}, \quad \text{.....(3)}$$

$$\phi = \alpha.\pi/2, \quad \text{.....(4)}$$

where z is the impedance, ϕ the phase angle and α a constant having the value $1 - m$ (equation (1)).

For a condition of constant phase angle, a log log plot of impedance against frequency should, therefore, also give a straight line of slope α.

Inasmuch as the impedance of the 6 cm.^{2} skin area under electrode A may be determined with the same arrangement of electrodes used for measuring the phase angle, it was considered to be of interest to make impedance readings at a number of frequencies (1) to determine with certainty that a constant phase angle condition existed above 11,000 c./sec., (2) to check the absolute values found by the direct method and (3) to obtain data at frequencies below 10,000 c./sec.[1]

Since it would be impracticable to measure impedances over a frequency range in as large a number of subjects as were used in the direct method (120), the number of subjects was limited to 40 (20 males and 20 females) and impedance readings were made at frequencies of 2000, 5000, 11,160 and 41,800 c./sec.

In making measurements, current was fed between electrodes A and C through a 20,000 ohm current limiting resistance and the voltage drop

[1] The phasemeters described are designed for operation at fixed frequencies. To investigate the low frequency range, special instruments would have had to be designed for each frequency investigated.

between electrodes B and A was determined by means of a vacuum tube voltmeter calibrated in ohms at each of the frequencies used.

Results. The measured impedances for these 40 subjects were found to fall on straight lines whose average slope (0·80) corresponds to a phase angle of 72°. The averages of the impedances at each frequency for all of these subjects are plotted in Fig. 6 and lie on a straight line differing but little in slope (0·79) from the average of the individual slopes.

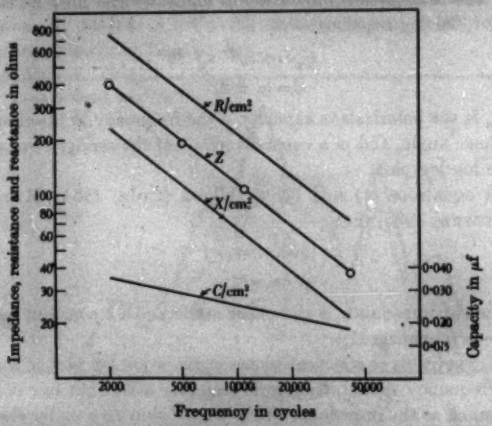

Fig. 6. Impedance components of the skin as a function of frequency. Z, average impedances of 40 subjects; electrode area 6 cm.^{2} R, X and C, resistance, reactance and capacity per unit area of normal human skin. The curves for R and C are to scale; the X values have been divided by 10. The values of R and C are calculated for an equivalent series circuit.

The resistance, reactance and capacity of the skin per cm.^{2}, being of basic interest, have been calculated from the average normal phase angle (71·3°) and the average impedance at each frequency, and are also represented in Fig. 6. It will be noted that the slope of the capacity-frequency curve is different from that of the corresponding curves for resistance, reactance and impedance (equations (2) and (4)).

In order to verify by direct measurement the constancy of phase angle in the lower frequency range (2000–11,160 c./sec.) indicated by the linearity of the log (impedance)-log (frequency) curve, phase angle determinations were made on two subjects over the entire frequency range i.e. at 2000, 5000, 11,160 and 41,800 c./sec. Since a phasemeter

operating at 2000 and 5000 c./sec. was not available for this purpose, readings were obtained at these frequencies by connecting current-carrying electrodes *A* and *C* in series with a resistance of 400 ohms, feeding the difference of potential across the resistance to one pair of plates of a cathode ray tube and simultaneously throwing the voltages tapped off between electrodes *B* and *A* across the other pair of plates; the phase angle being calculated from the form of the ellipse so produced. Phase readings made in this way were accurate to ± 3 p.c.

The phase angle values obtained for both of these subjects showed variations of less than $\pm 2°$ over the entire frequency range indicated and confirmed the constancy indicated by the linearity of the impedance-frequency curve.

The absolute value of the phase angle and its constancy with frequency as measured by the direct and indirect methods are in close agreement. The results obtained may, therefore, be taken to represent within the experimental error the average phase angle properties of normal skin over the frequency range investigated.

Comparative experimental analysis of the band, concentric and two-electrode techniques

In view of the fact that the phase angles reported here differ from those hitherto obtained (1) in remaining constant with frequency and (2) in showing values some 15–20° higher at 10,000 c./sec. than those reported by Hozawa and by Horton & van Ravenswaay, it was considered important to examine the techniques used by previous authors in order to determine wherein they were at fault.

The band-electrode method. The electrode set-up for this method described by Horton & van Ravenswaay is shown in Fig. 3*b*. As has already been pointed out, the two edges *a* and *b* of current-carrying electrode *A* are at a different potential with respect to voltage-tapping electrode *B* which is placed to one side. It would, therefore, be expected that the magnitude of the error introduced in the measurement of impedance components or phase angle would increase (1) with the width of current-carrying electrode *A*, (2) with the separation of electrodes *A* and *B* and (3) with the frequency.

The possibility of the measured phase angle decreasing with increased electrode width was indicated in the work of the authors of this method. Their measurements with an electrode 1·5 cm. wide at 10,000 c./sec. gave a value of 54° whereas with an electrode of twice this width (3 cm.), a phase angle of about 40° was found at the same frequency. (It is not

stated whether or not these measurements were made on the same individual.) To determine the effect of varying electrode widths, a series of measurements was made of the phase angle of the skin area under electrode *A* with electrodes 2, 10 and 20 mm. wide (Fig. 3*b*), voltage-tapping electrode *B* being maintained first a fixed distance and then, at varying distances, from *A*. The 10 and 20 mm. electrodes were made of sheet tin covered with flannel and soaked in 1 p.c. saline. The 2 mm. electrode was a flattened band of solder wire without any covering,

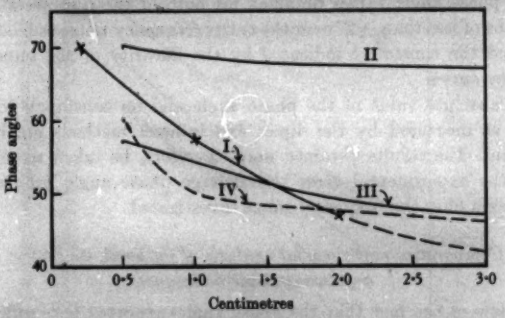

Fig. 7. Effect of electrode size, position and spacing on the measured phase angle. Curve I. Band electrode technique: effect of varying the width of current electrode *A* (Fig. 3*b*) alone; electrode *B* at a constant distance of 5 mm.; abscissæ, width of band *A*, ordinates, phase angles. Curve II. Band electrode technique: effect of varying the spacing between bands *A* and *B*; electrode *A*, 2 mm.; abscissæ, distances between electrodes, ordinates as in I. Curve III. Similar to curve II, band *A*, 10 mm. wide. Curve IV. Concentric electrode technique: effect of displacement of electrode *B* from inside to positions at various distances outside annulus *A*; abscissæ, distances from annulus, ordinates as in I. The displacements of electrode *B* were made towards the left of the position shown in Fig. 3*c*. Measurements similar to curves II and III with electrode *A* 20 mm. wide gave values below the range of the phasemeter (45°) at distances of 1 cm. or more.

fastened over the saline moistened skin. All measurements were made at 15,300 c./sec. using the phasemeter described. The results are shown in Fig. 7.

With voltage-tapping electrode *B* at a fixed distance of 5 mm., the phase angle values diminish rapidly with increasing electrode width, dropping sharply from 70° for the 2 mm. electrode to 47° for one of 20 mm. (curve I). Attempts were made to obtain measurements with an electrode 30 mm. wide, but the phase angle fell below the range of the phasemeter which was calibrated for values between 45° and 90°. By

extrapolating along curve I a value of 42° is obtained. The phase angle of the skin of this same subject measured with a concentric electrode was found to be 73°.

The effect of increasing the spacing between electrodes *A* and *B* is shown in curves II and III. The phase angle decreases with increasing electrode separations up to 30 mm., the rate of decrease being markedly less for a narrow than for a wide electrode. No significant differences in the phase angle values were noted using voltage-tapping electrodes of 2 and 10 mm. width either when the width of electrode *A* was varied alone or when the electrode separation was varied keeping the width of electrode *A* constant. It will be noted that the results obtained with a bare metal 2 mm. electrode, 70°, are closest to those for the concentric electrode (73°).

Comparative experiments were also carried out using concentric electrodes *A–B* to determine the effect (1) of varying the direction of current flow and (2) of shifting voltage tap *B* from inside to outside the annulus. The experimental set-up is shown in Fig. 3*c*. No significant difference in phase angle was obtained by shifting current electrode *C* from a position opposite electrode *A* (dotted line position) to a position 10 cm. along the arm on the same side (full line position). With electrodes *C* and *A*, in this latter position, the effect of shifting voltage-tapping electrode *B* from inside to a position 5 mm. outside annulus *A* was to drop the measured phase angle from 73° to 60° and, as electrode *B* was moved away from the periphery of the annulus, the decrease in phase angle shown in curve IV was obtained.

The effect of changes in frequency on the phase readings may be deduced from the results reported by Horton & van Ravenswaay. Using a current electrode 3 cm. wide, these authors give a reactance-frequency curve which, when plotted on log log co-ordinates as in Fig. 8, is substantially linear between 200 and 2000 c./sec. but which diminishes in slope at frequencies lying both below and above this range. From the Fricke relation, it follows that the phase angle should remain constant over the linear range. The slope of the linear portion is 0·78, giving a phase angle of 70°. This value is of the order of those found with concentric electrodes. The non-linearity of the reactance-frequency curve at low frequencies may be due to an actual change in the phase angle of the skin. At frequencies above 2000 c./sec. the non-linearity of the curve may be explained on the basis of a progressive increase in the potential gradient across the electrode. The work of Horton & van Ravenswaay indicates that the impedance of the deep tissues (inner impedance)

remains substantially constant at frequencies up to 10,000 c./sec. It can be shown that, in an infinite network composed of an impedance of constant value (deep tissue) shunted by a series of condensers (the skin), the potential gradient will tend towards zero as the frequency is decreased.

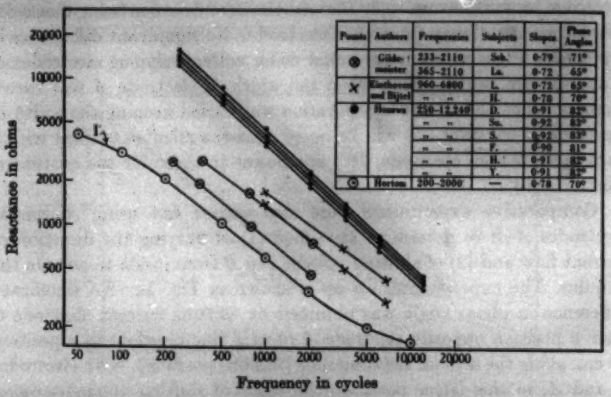

Fig. 8. Reactance as a function of frequency according to various authors. The curves are identified in the chart. The results for all normal subjects reported in the literature (except as indicated below) are given in which measurements were made of the skin of the *arm* at more than two frequencies. The curves for two of the subjects of Hozawa are not plotted. They fall on or across the four upper curves and have been omitted to avoid confusion. The curves omitted are also linear. Their slopes are included in the chart. The reactance values in curve I have been multiplied by 10.

The two-electrode method. The technique of Hozawa will be taken as illustrative of this method. This author uses two platinum electrodes of 4·2 cm.^{2} area spaced at a distance of 10 cm. along the forearm, the upper electrode lying just below the bend of the elbow. It can be shown that the measurements of the impedance properties of the "skin" made in this way include a large deep tissue component. To do this, two concentric electrodes are mounted on the forearm as shown in Fig. 3*f* in the positions indicated by Hozawa. Current is fed from annulus *A* to annulus *A'* and voltages are tapped from the two centre electrodes *B*, *B'*. The impedance corresponding to this difference of potential and the phase relation between current and voltage may then be determined by means of an A.C. comparator described elsewhere [Bagno & Barnett, 1938]. The values obtained represent the properties of the deep tissues,

the effect of the skin being eliminated. In six subjects measured in this way at 11,160 c./sec., the impedances varied from 102 to 190 ohms depending on the arm size and the amount of subcutaneous fat [Barnett, 1937] and the Q-factor from 0·22 to 0·08. By tapping voltages from electrode B and its corresponding annulus A instead of from B and B' (Fig. 3*c*, full line position) the phase angle and impedance of the skin area under the annulus may be determined without disturbing the electrodes. Finally, the phase angle of the skin and the deep tissues together may be measured by connecting annuli A and A' across the terminals of a phasemeter designed for two-electrode operation [Barnett, 1935]. In a typical instance in which the phase angle and the impedance of a 6 cm.^{2} skin area under one annulus were 71·5° and 125 ohms respectively at 11,160 c./sec., the corresponding values for the deep tissues lying between the electrodes were 6·4° and 104 ohms. The measured overall phase angle for all the anatomic elements between electrodes (skin—deep tissues—skin) was found to be 49·0° which is in good agreement with the value calculated from the impedance properties of the skin and the deep tissues taken separately (48·7°). These values are of the order of those reported by Hozawa at 12,240 c./sec. It is obvious that skin phase angle determinations made by two-electrode techniques are subject to serious error due to the impossibility of excluding the effect of deep tissues. In the particular case given, this error exceeded 20°. Since the impedance of the skin decreases with frequency (Fig. 6) while that of the deep tissues remains relatively constant [Horton & van Ravenswaay], the magnitude of the error introduced by the deep tissues should increase with frequency. It is, probably, for this reason that the phase angle-frequency curves of Hozawa, Gildemeister and Einthoven & Bijtel show a decreasing characteristic with increasing frequency. The experimental data of Gildemeister and of Einthoven & Bijtel permit an estimate of the phase angle of the skin independently of the deep tissue impedance. It will be shown further on that, by excluding this impedance, phase angles calculated from their skin data become constant with frequency and show an absolute value corresponding closely to those obtained by the concentric electrode technique.

Discussion

The measurements made here represent, strictly speaking, the properties of the "surface sheath". While the depth of this sheath has not yet been directly measured, there are reasons for believing that it represents only the epidermal layers of the skin. The impedance of blood

[Crile *et al.* 1922] and of highly vascular tissues such as muscle [Eyster *et al.* 1933] is known to be relatively low. The epidermis is substantially avascular and this would tend to make it a poor conductor. The presence of a capillary bed just beneath the epidermis (in the corium) would appear to provide a conducting sheath of low impedance forming a sharp electrical boundary for the poorly conducting surface sheath. This view is in accord with those of previous workers in this field. Gildemeister, Einthoven & Bijtel, and Hozawa interpret their measurements of the "skin" as representing the properties of the epidermis. Although only two electrode techniques were available to these authors, it was recognized by all of them that a clear distinction must be made between the properties of subepidermal structures and those of the epidermis itself. In tapping off voltages through the skin, the epidermis appears to function as a relatively high impedance layer interposed between the voltage-tapping electrode and the more highly conducting underlying structures composed of the derm proper, the subcutaneous tissues and the muscles.

It has already been shown that the linear portion of the log (reactance) log (frequency) curve of Horton & van Ravenswaay (curve I, Fig. 8) indicates the existence of a condition of constant phase angle over the range 200–2000 cycles and an absolute value of 70° within this range for the single subject measured. It was thought to be of interest to make corresponding log log plots from the reactance data of Gildemeister, Einthoven & Bijtel and Hozawa for purposes of comparison. The curves obtained are shown in Fig. 8. They have a striking common feature. All of the curves are linear over the entire range of frequencies employed. The linear slopes and corresponding phase angles (equation 4) are tabulated in Fig. 8. The four subjects of Gildemeister and of Einthoven & Bijtel show phase angles (65–71°) falling within the limits of the distribution curve in Fig. 5. Those of Hozawa run consistently higher, with a mean of 82°. There is no apparent explanation for the elevated results obtained by this author. They may, possibly, be due to the race of his subjects (Japanese).

The discrepancies between the phase angles calculated directly from resistance and capacity values at each frequency (Fig. 1) and those obtained above by use of the Fricke relation from the reactance-frequency data alone are easily explained. In the former case, the phase angle of deep tissue being small (curve VI, Fig. 1), substantially all of the deep tissue impedance is included as resistance in the calculations and introduces an error which, it has already been shown, increases with frequency.

The reactive component of the deep tissue impedance, on the contrary, is negligibly small in comparison with that of the skin. The measured reactances, therefore, represent closely the properties of the skin itself.

Analysed in this way, the data obtained by two-electrode techniques are seen to be in excellent agreement with the phase angle findings reported herein both as to constancy with frequency and, the results of Hozawa excepted, as to absolute value.

It is important to correct a systematic error which has appeared in previous work relative to the calculation of the polarization phase angle of normal human skin. The Fricke relation was published in 1932. Prior to that time, following the initiative of Wien [1896], it was customary to calculate the cotangent of the phase angle, ϕ, of a polarization impedance from the formula

$$\cot \phi = 2\pi \times (\text{frequency}) \times (\text{capacity}) \times (\Delta R), \qquad \ldots\ldots(6)$$

where ΔR represented the difference between the measured resistance and the resistance at infinite frequency. For metals dipping into solutions, the cotangent values frequently remain constant with frequency. The infinite frequency value was generally taken as the impedance measured at about 100,000 c./sec. or estimated from the intercept on the resistance axis at zero reactance of the resistance-reactance curve. Gildemeister, in 1928, using this method, found cot ϕ, calculated from his own data and that of Einthoven & Bijtel, to remain substantially constant with frequency. This constancy, it can be shown, is the result of a fortunate choice of frequency (100,000–150,000 c./sec.) for estimating the infinite frequency resistance. The deep tissues cannot properly be considered as purely or even approximately resistive at 100,000–150,000 c./sec. as in the case of solutions. Measurements by the present author above the frequency range investigated by Horton & van Ravenswaay show the phase angle of the inner impedance to increase continuously up to 126,000 c./sec. (curve VI, Fig. 1) when measured along the arm axis. Transversely through the arm (Barnett, 1937), the phase angle of deep tissue may be as much as three times the longitudinal value and has been found to have a more steeply rising frequency characteristic. It follows therefore that (1) to obtain a proper infinite frequency value for the skin itself, measurements would have to be made at millions of cycles, and (2) as higher and higher frequencies are employed, ΔR will increase continuously and cot ϕ will be less and less constant with frequency. It is probably because of the impossibility of accurately estimating the infinite frequency resistance from existing data that Cole's calculations [1933] based on the measurements of Gildemeister and of Einthoven & Bijtel give a polarization phase angle of only 55°.

Cole [1932] has made explicit reservations regarding the applicability of the network shown in Fig. 2*a* to tissues. He and Curtis [1936] have been able to show that, in the case of sheathed structures such as the sciatic nerve of the frog and the cat, at least two variable impedance elements are present, one accounting for the properties of the sheath and the other for the bundle of nerve fibres inside the latter. There is a possibility that the same is true for muscle sheaths and the muscle fibres contained therein. In the case of biological materials such as suspensions of red cells and marine eggs, where an enclosing sheath is absent, the impedance properties of all the cells are well represented up to high frequencies by a

network containing a single variable impedance element of constant phase angle. This may be due to the fact that the individual cellular elements are nearly alike and widely spaced. In the case of the skin, the various cellular layers composing the epidermis are packed closely together and change progressively from a wet type in the stratum germinativum to a relatively dry type in the higher layers. The cells in the upper tiers must, obviously, differ in their electrical properties from those lower down. It might, therefore, be expected that, for the skin, one must postulate as many different variable impedance elements as there are types of cells. The experimental data here presented indicate, nevertheless, that the properties of the skin over the range 2000–40,000 c./sec. may be represented by a single bare variable impedance element of constant phase angle as shown in Fig. 2*b*. The analysis of the work of previous authors (Fig. 8) makes it probable that this simple network is valid down to about 200 c./sec. At frequencies below 200 c./sec., the measurements of Horton & van Ravenswaay appear to indicate a progressive decrease in phase angle (Fig. 8). This can be accounted for, as shown in Fig. 2*c*, by the intervention of the leakage resistance of the skin, R_1, which is known to be of the order of 75,000 ohms/cm.^{2} for most normal individuals [Einthoven & Bijtel]. The network represented in this latter figure may, therefore, be taken, provisionally, to summarize the impedance properties of the skin from zero to 40,000 c./sec.

The extreme simplicity of this network is very striking considering the heterogeneity of the cellular aggregate of which the epidermis is composed. Blinks [1933] has suggested that, in tissues where the cellular elements are non-spherical and closely packed, the possible intervention of distributed capacities such as are found in cables is to be considered. It is known that distributed networks of this kind may be arranged to give capacities varying with frequency in the same way as the single variable impedance polarization element postulated by Cole. It would seem hazardous, at the present time, to speculate as to whether the constant phase angle of the skin is due to polarization effects or distributed capacities or both. Nevertheless, as throwing possible light on an eventual choice, it may be of interest to note, that, in four different types of cases in which polarization phenomena are observed—the skin, metals dipping into solutions, cuprous oxide rectifiers and collodion-solution interfaces—a structural gradient either is known, or appears, to exist. In the case of the skin, it is the superposed strata of the epidermis. At the surface of metals in contact with solutions, the diffuse double layer is generally assumed to present such a gradient [Muller, 1933]. For cuprous oxide

rectifiers, the gradient appears at the transition zone between the pure copper on one side and the pure cuprous oxide on the other [Grondahl, 1933]. Wilbrandt [1934] and Spiegel-Adolf [1937] have reported more marked polarization properties for partially dried collodion than for the completely dry membrane. The drying collodion should present a gradient due to evaporation from the surface. In the finally dried membrane, this gradient would be greatly diminished or even destroyed.

The nature of the gradient in these various cases has not yet been determined and may be different in each case. For the skin and a number of metal-solution interfaces [Fricke], the Fricke relation has been shown to hold over a certain frequency range. Mathematical analysis and experimental evidence [Muller] point to a probable exponential type of gradient both as to potential and charge distribution in the diffuse double layer. The charges of opposite sign in this layer may be treated as condensers. Methods for calculating the properties of electrical networks made up of condensers and resistances and having exponential and other types of gradients are well known. Preliminary studies of various networks with exponential gradients have been made with a view to determining whether or not a constant phase angle characteristic may be obtained. It has been found that, in an infinite network composed of a series of condensers each shunted by a resistance, if the resistances change exponentially with relation to the capacity, the phase angle at the terminals rises from zero at zero frequency to a plateau value which remains constant over a very wide frequency range. The detailed analysis of this and similar networks will be presented in a separate paper wherein the question of their possible use as a representation of the properties of the skin and other structures showing polarization phenomena will be taken up.

The problems relative to the measurement of the impedance properties of sheathed structures such as nerve trunks, nerve fibres (and possibly isolated muscle) are very analogous to those presented by the skin and the deep tissues. The phase angle of frog nerve sheath (sciatic) has been found to be 49–64° [Cole & Curtis, 1936]. In studies of nerve impedance made in the past, two electrode techniques have been used exclusively. It now appears that the measurements made represent largely the properties of the sheath, the high reactance of the latter at low and intermediate frequencies masking the low impedance properties of the nerve fibre bundle up to relatively high frequencies. Obviously, the properties of the nerve bundle and of its individual fibres are of greater interest than that of the sheath, and four electrode techniques would appear to offer a ready

means for traversing the latter. It is believed that the experimental studies of the band, concentric and two-electrode methods here reported may prove of value in the critical evaluation of results already obtained and in the solution of similar impedance problems which present themselves in this related field.

Summary and conclusions

1. An improved three-electrode technique for the measurement of the phase angle of the skin is described involving the use of a new type of direct-reading phasemeter and a special concentric electrode.

2. The phase angle of the skin in 120 normal subjects as measured with a phasemeter is found to vary between 64° and 78° at 15,300 cycles with a mean value of 71·3° and a standard deviation of 2·85°.

3. Impedance measurements over the frequency range 2000–41,800 cycles confirm the phase angle values found with a phasemeter.

4. The phase angle of the skin is shown to remain constant with frequency by direct measurement. The form of the impedance-frequency curve confirms the existence of this condition.

I am indebted to Mr S. Bagno for the construction of instruments and valuable aid in the course of this work.

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J. Physiol. (1936) 93, 367-381 612.352.2

A STUDY OF THE SOURCE OF LIVER FAT USING DEUTERIUM AS AN INDICATOR

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(*Received* 1 *June* 1938)

One of the many problems arising out of the recent researches carried out in the Department of Physiology and in this department is the source of the fat which accumulates in the liver when animals are maintained on a diet poor in lipotropic factors and when certain extracts of the anterior pituitary gland are administered. This problem may be attacked by the use of "labelled" fatty acids. While there are several methods of preparing these "ear-marked" molecules, none of these possesses all the advantages which may be secured by the use of deuterium.

This communication consists of two parts. In the first a micro-density method for the determination of deuterium oxide, elaborated by one of us (H.M.B.), is described. In the second part the results of the application of this method to certain problems of fat mobilization are presented and discussed.

Part I. A micro-density method for the determination of deuterium oxide

Rittenberg & Schoenheimer [1935] have described methods for the use of deuterium-containing molecules in studies of intermediary metabolism. To determine the deuterium content of water arising from the combustion of organic material, they utilized both the interferometer method of Crist *et al.* [1934] and the Richards [1912] density float. The first of these methods requires costly equipment, the second is unnecessarily sensitive for most of the analyses encountered in our investigations. For these reasons a new procedure was developed.

The apparatus is shown in Pl. I, fig. 1. (*a*) is the tube in which the water obtained from the combustion of deuterium-containing material is collected after final purification. The sample of water, which amounted

usually to 0·3 g., is transferred by means of the pipette (*b*) to the container (*c*). The pycnometer bulb (*d*) was made of soft glass and had a capacity of approximately 0·26 g. The bulb of the pycnometer terminated in a very fine capillary tube (diam. of bore 0·003–0·006 in.) which was bent to form an angle of about 30° with the bulb. With the capillary tube immersed in the container, the bulb is heated; on cooling, the bulb partially fills. By repeating this process the last traces of air can be removed. The heating is accomplished by means of the electric resistance heater (*e*). This consists of a piece of pyrex glass tubing, of slightly smaller diameter than the bulb, wound with a few turns of nichrome wire.

When the pycnometer bulb has been completely freed of air bubbles the bulb is cooled, under running tap water, below room temperature, the capillary being still immersed in the sample, so that on its removal no air will suck back. The bulb is now clamped in the rubber-covered artery clamp (*f*) which is slipped over a piece of stiff wire. The wire is set in a thermos flask partially filled with water the temperature of which has been adjusted to 32·00° C. Care is taken to ensure that the top of the capillary is not below the surface of the water. The top of the thermos flask is closed with a plug of cotton. After 4 or 5 min., temperature equilibrium between the content of the pycnometer and the water in the flask having been reached, the top of the capillary is touched with a piece of filter paper to remove water which has been expelled. The bulb is then removed and wiped dry with chamois. Care is taken never to touch the bulb with the hands.

At first the pycnometer was weighed on an ordinary Kuhlmann microbalance. It was found that repeated fillings could be duplicated within $\pm 15\gamma$ which was about the accuracy with which duplicate weighings on the empty bulb could be made. (A second bulb of approximately the same shape and size was used as a counterweight.) These weighings were tedious and time-consuming and a microbalance of a different design was found to be preferable.

A torsion microbalance

The balance to be described is similar to one first constructed some years ago for use in adsorption measurements in the Department of Chemistry of this University. Grateful acknowledgement is made to Prof. F. B. Kenrick of that Department, under whose direction the original balance was constructed.

A photograph of the balance is shown in Pl. I, fig. 2. The support is made of $\frac{1}{4}$ in. glass rod. The balance beam is constructed as follows. Thin-walled tubes of about 2 mm. diameter are drawn out from $6 \times \frac{1}{2}$ in. soft glass test-tubes. Two of these tubes are joined by a 4 cm.

Fig. 1. Apparatus for density determination.

Fig. 2. Torsion microbalance.

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length of glass rod of the same diameter. A U-shaped arch is sealed to this rod to prevent the beam from sagging when the seal is made to the centre suspension. At either end of the beam small forks of glass rod are sealed; these serve to hold the basket suspensions. A glass pointer is sealed to one of the forks. The suspensions are made from 0·0006 in. diam. tungsten wire and are sealed directly to the glass. Hooks constructed from 0·003 in. tungsten wire are sealed by small lumps of glass to the centre of each of the end suspensions. The bends in the hooks are at right angles to the balance beam, i.e. parallel to the suspensions.

Two glass baskets are constructed by blowing thin-walled bulbs approximately 2·5 cm. in diameter and flattening them in the flame. The unwanted part of the bulb is cut away with a fine flame. Supports made from glass rod about 1 mm. diam. are sealed to the baskets; these supports are bent at the top to give circular openings to support the pycnometer bulb and the counterweight respectively.

It is necessary to exercise great care in sealing the suspensions to the glass to prevent the flame from coming in direct contact with the tungsten wire as the resulting oxidation would weaken it tremendously. To secure a flame sufficiently fine to prevent this a blow torch is constructed from pyrex glass in which the opening in the end is approximately 2 mm. Air is admitted to the torch through a fine capillary.

The balance is placed in a wooden box thermally insulated with 2 in. of ground cork. Double doors, each fitted with a double thickness of glass, are tightly fitted into felt weather-stripping. These precautions are necessary to prevent convection currents from affecting the balance.

The balance stand is fastened to the base with sealing-wax. A millimetre scale is ruled on cardboard, which is glued to a piece of mirror. The centre of curvature of the scale is adjusted to coincide with the centre of suspension of the balance.

The pycnometer bulb, filled at 32·00° C. with water distilled from alkaline potassium permanganate and phosphorus pentoxide, is placed on the left-hand basket and an empty bulb of similar size but of somewhat greater weight on the right-hand basket. Using broad-tipped forceps the baskets are hooked to the end suspensions and the two sides are balanced by adding glass to the baskets.

Weights are constructed by cutting 6·0 cm. lengths of the tungsten wire used for the suspensions. Several determinations of the weight of 25·0 cm. lengths of this wire showed it to be remarkably uniform. 25·0 cm. weighed $1100 \pm 5\gamma$. One 6·0 cm. length therefore weighed 264γ. Eight of these 6·0 cm. lengths were placed on the basket supporting the pycnometer bulb.

The centre of gravity of the balance is adjusted by bending the balance beams, final adjustment being made by sealing small lumps of glass to the central arch of the beam. The centre of gravity is adjusted so that 264γ causes a deflexion of 40–50 divisions on the scale. At this sensitivity the deflexion is proportional to the increase in weight for deflexions of 35 divisions on either side of the position where the balance beam is parallel to the floor. This is determined by adding successive 1·0 cm. lengths of the tungsten wire; at positions near the limits of swing of the balance the sensitivity becomes somewhat greater. Readings are therefore always taken within ± 35 divisions of the parallel position of the balance beam. Each of the eight weights gave deflexions equal to $44 \pm \frac{1}{2}$ divisions. 1 mm. deflexion was therefore equal to about 6γ.

The capacity of the pycnometer bulb filled at 32·00° C. with redistilled water equals 0·263450 g. 100 p.c. deuterium oxide has a density 1·1078 times that of ordinary water at 32·00° C. (calculated from the figure of 1·1074 25°/25° of Tronstad *et al.* [1935] and Lewis & Macdonald's [1933] data for the change of volume of deuterium oxide with temperature). The difference in weight between the bulb filled with 100 p.c. deuterium oxide and redistilled tap water equals $0{\cdot}1078 \times 0{\cdot}263450 = 28{,}400\gamma$.

The percentage of deuterium oxide by weight in an unknown sample is therefore

$$\frac{\Delta s}{28{,}400} \times \frac{(0{\cdot}263450 \times 1{\cdot}1078)}{(0{\cdot}263450 + \Delta s)},$$

where Δs equals the difference in weight in γ between the unknown sample and redistilled ordinary water. The deflexion produced by 1–6 cm. length of wire ($44 \pm \frac{1}{4}$ divisions) is equivalent to 1·02 p.c. deuterium oxide. One division on the scale therefore corresponds to 0·0232 p.c. deuterium oxide.

Due to electrostatic charges introduced on the bulb when it was wiped, the reading indicated by the pointer when it has stopped swinging invariably changes from 3 to 5 divisions. For this reason the final reading is taken $\frac{1}{2}$–1 hr. after the baskets have been suspended from the beam. This reading is perfectly reproducible within $\pm\frac{1}{4}$ division. The shift is invariably greater on days of low humidity. In the summer the reading became constant within about 15 min.

The balance zero, i.e. the pointer reading when the pycnometer is filled with redistilled ordinary water, did not shift more than 2 divisions during a period of months. As a rule, this zero was checked every 3 or 4 days or, when samples of very low deuterium content were being analysed, daily. Evaporation of water from the capillary of the pycnometer bulb was very slow. The change in weight from this cause amounted to from 1 to 3 divisions in 24 hr., depending on the room temperature. This loss was, of course, negligible in the time required to make a weighing. Duplicate fillings of the pycnometer from the same water sample checked within ± 1 division.

The effect of variation of temperature of the water in the thermos flask in which the pycnometer was immersed amounts to 13 divisions per 1·00° C. change. This observed change is identical with the change calculated from the alteration in density of water with temperature corrected for the cubical expansion of the pycnometer bulb. As this temperature is maintained to $32{\cdot}00 \pm 0{\cdot}02$° C. the error from this cause amounts to less than $\frac{1}{4}$ division.

TABLE I. Solutions of deuterium oxide and water prepared from 99·6 p.c. deuterium oxide

Prepared concentration D_2O p.c.	Concentration found D_2O p.c.
6·31	6·31
2·98	3·00–2·94*
2·61	2·53
1·47	1·48
1·11	1·11
0·98	0·94–0·96*
0·52	0·47–0·48*
0·29	0·29–0·27*
0·08	0·07

* Duplicate analyses.

Table I shows the results of a series of analyses carried out on prepared dilutions of 99·6 p.c. deuterium oxide with redistilled ordinary water. Duplicate determinations on the same sample have an error no greater than $\pm 0{\cdot}03$ p.c. deuterium oxide. As the sample used for analysis is less than 0·3 g. this accuracy compares favourably with that attainable with the interferometer using a sample of similar size.

The procedures necessary for the analysis, with the exception of the weighing, can be carried out in approximately 15 min.

Part II. The source of the excess fat which accumulates in the liver[1]

Experimental methods

In the experiments to be described the procedure consisted in feeding large groups of female mice on the following diet: meat powder 20 p.c. by weight, brown bread 37 p.c., linseed oil (containing from 5 to 7 atoms p.c. of deuterium introduced by means of a palladium catalyst) 15 p.c., "purina" (commercial mixed diet) 10 p.c., sucrose 10 p.c., yeast 4 p.c. and salt mixture (McCollum's No. 185) 4 p.c. Vitamins A and D were added and 25 mg. of choline for each 4 g. of food were provided. In some experiments the diet included 20 p.c. of linseed oil containing deuterium and only 15 p.c. of meat powder. The feeding periods varied from 9 to 14 days as indicated in the tables. The average food consumption was approximately 3 g. per mouse per day. The mice were kept in individual cages in a room in which the temperature was maintained at approximately 28° C. At the end of the feeding period on the above diet, groups of mice were killed to secure control values. The livers from seven to ten mice were pooled for an analysis. Fatty acids were estimated by the direct saponification procedure. In the experiments in which neutral fat and phospholipin fractions were separated, fatty acid determinations were made on the ether-soluble fractions by the method described previously [Best *et al.* 1934]. After discarding the gastro-intestinal tract, the carcasses were treated in a similar manner. From 0·3 to 0·4 g. of fat from each sample was analysed for its deuterium content following Rittenberg & Schoenheimer's [1935] procedure for combustion and purification and utilizing the density determination method already described for the final analysis. The same procedure was applied to the livers and carcasses of the test mice which, after the control period, had been subjected to various experimental procedures such as starvation, maintenance on a high-carbohydrate or a high-protein diet, injection of anterior pituitary extract or exposure to carbon tetrachloride vapour. Comparison of the deuterium content of the fat from the livers and depots of these animals with the corresponding values from the control groups indicated the source of the excess fat in the liver.

[1] A preliminary note on this subject appeared in the *Proceedings of the American Society of Biological Chemists* (*J. biol. Chem.* **123**, iii, 1938).

When sufficient fat was available, duplicate saponifications were carried out. Estimations of the deuterium content of the fats were made in duplicate or triplicate. The agreement among determinations was excellent and only average values have, for the most part, been recorded in the tables. The deuterium content of the body water was always determined [Schoenheimer & Rittenberg, 1935] as this gave some indication of the amount of deuterium-containing fat being utilized by the animal at that particular time.

Since it was known that the increase in fat that occurs in the livers of animals is, under many conditions, largely neutral fat, it was important to determine the distribution of deuterium between the phospholipin and neutral fat fractions. In a number of groups of animals separation of these components of the depot fat was therefore effected. This procedure could not readily be extended to the livers in the experiments on mice as insufficient fat was available.

Experimental results and comments

The source of increase in liver fat in starvation.

The results of four experiments in which mice were starved for various periods are summarized in Table II. The duration of the initial feeding period in which linseed oil containing deuterium was provided and the deuterium content of the oil are indicated in each case.

In Exp. 1, for an unknown reason, the expected accumulation of fat did not occur in the livers of the mice starved either for 24 or 32 hr. On the other hand, the deuterium content of the liver fat of the group starved for 32 hr. became essentially the same as that of the depot fat. The results of this experiment provide evidence that there is an interchange of fat between the liver and depots under these conditions. Since there is so much more fat present in the depots than in the liver, transport of liver fat with its somewhat higher deuterium content to the depots would not be detected by an increase in percentage of deuterium in depot fat. Equilibrium between the deuterium content of liver and depot fat is taken as conclusive evidence of an interchange of fat between these two sites.

In Exp. 2 the amount of deuterium in the liver fat of the control animals at the conclusion of 9 days' feeding is almost the same as that found in the liver fat of the control group of Exp. 1. The deuterium content of the depot fat of these animals is, however, significantly lower than in the first experiment; this is undoubtedly due, in part, to the shorter feeding period. After 48 hr. starvation the liver fat has nearly tripled

TABLE II. Fasting

Exp.	Group	No. of animals	Liver fat p.c.	Weight of liver fat per mouse g.	D_2 in liver fat atoms p.c.	Av. D_2 in liver fat atoms p.c.	Weight of depot fat per mouse g.	D_2 in depot fat atoms p.c.	Av. D_2 in depot fat atoms p.c.	D_2O in body water p.c.
1	Controls fed 13 days on diet with 15 p.c. linseed oil containing 5·90 atoms p.c. deuterium	9	4·5	0·051	2·67	2·55	2·6	1·88, 1·94	Total 1·91	0·43
		10	4·7	0·058	2·43				Neutral fat 1·78 Phospholipin 2·10	
	Fasted 24 hr.	10	5·5	0·046	2·17	2·17	2·7	1·84, 1·82	Total 1·83	0·54
									Neutral fat 1·85 Phospholipin 1·93	
	Fasted 32 hr.	10	4·6	0·046	2·06	2·06	2·5	2·04, 2·09	Total 2·07	0·37
									Neutral fat 2·08 Phospholipin 1·96	
	Injection of anterior pituitary extract. Killed after 7 hr. fasting	10	10·8	0·153	2·06	2·04	2·9	1·98, 1·98	Total 1·98	0·38
		10	9·5	0·131	2·02				Neutral fat 2·01 Phospholipin 1·79	
2	Controls fed 9 days on diet with 15 p.c. linseed oil containing 5·90 atoms p.c. deuterium	15	2·9	0·037	2·38, 2·38	2·38	2·8	1·05, 1·04	1·05	0·11
	Fasted 48 hr.	15	9·9	0·092	1·25, 1·22	1·24	2·3	0·92, 0·92	0·92	0·10
3	Controls fed 14 days on diet with 20 p.c. linseed oil containing 6·51 atoms p.c. deuterium	8	4·4	0·036	3·92	3·80	4·5	2·91	2·80	0·54
		7	4·3	0·037	3·67		5·3	2·69		0·43
	Fasted 52 hr.	8	12·7	0·091	2·74	2·70	3·8	2·67, 2·71	2·56	0·62
		7	12·8	0·096	2·65		3·4	2·40, 2·47		0·66
4	Controls fed 14 days on diet with 15 p.c. linseed oil containing 4·90 atoms p.c. deuterium	8	5·8	0·061	2·06	2·08	6·2	2·03	2·05	0·23
		7	5·0	0·049	2·10		5·7	2·06		0·18
	Fasted 7 days	8	17·7	0·125	2·05	2·05	2·6	2·17	2·15	0·25
		7	17·2	0·115	2·04		2·3	2·13		0·27

in amount, at the same time the percentage of deuterium in the liver fat has decreased. It is still, however, considerably above that of the depots. Since there is such a great difference between the deuterium content of liver fat and depot fat in the control animals, no conclusions as to the source of fat increase in the starved animals can be reached from this experiment. As it has already been shown that an interchange of fat between liver and depots occurs on starvation, irrespective of any increase in total liver fat, it is not possible to decide how much of the decrease in deuterium percentage in the liver fat of the starved animals is due to this cause.

In Exp. 3 much more of the dietary fat was stored in the depots. The liver fat in the starved animals has again nearly tripled in amount. The deuterium content of the liver fat although lower than that of the control animals is almost the same as that of the depot fat. It is apparent that the increase in total fat in the livers of the starved animals must have arisen largely from a deuterium-containing source, that is, by transport of fat from the depots to the liver.

Exp. 4 in which the mice were starved 7 days confirms this result. Here no significant change occurred in the deuterium content of the liver fat of the starved animals although a tremendous increase in total fat resulted from the 7 days' starvation. This was the only experiment in the present investigation in which the deuterium content of the liver fat of the control animals was not significantly greater than that of the depot fat at the beginning of the fasting period. This result could be explained on the assumption that the control animals had not eaten for some time before they were killed. It will be remembered that the results of Exp. 1 showed that the depot fat and liver fat may become equal in deuterium content during starvation with no change in total liver fat.

The results of the deuterium analyses of the phospholipin and neutral fat fractions of the depot fat from the animals in Exp. 1 show considerable variation. No significant difference, however, in deuterium content appears to exist.

Injection of extract of the anterior pituitary gland.

Twenty-nine mice were given a subcutaneous injection of 15 mg. of extract prepared from the anterior lobes of ox pituitary glands [Best & Campbell, 1936]. These animals were fasted for 7 hr. after the injection and were then killed. The results are shown in Table II, Exp. 1. The injection of the extract has caused an extensive increase in liver fat. Although the deuterium level of the liver fat of these animals has fallen

below that of the control group, it is equal to that of the depot fat. It is apparent that the increase in liver fat resulted largely from a transport of fat from the depots.

Carbohydrate feeding.

Mice maintained on a high-carbohydrate diet poor in lipotropic factors develop fatty livers. Following the initial feeding period in which fat containing deuterium was provided, groups of mice were fed on the following diet: sucrose 76 p.c., agar 15 p.c., meat powder 5 p.c., salt mixture 4 p.c. and vitamins A, D and B_1. The experimental results are summarized in Table III. Since one group of animals served frequently as controls for several experiments of different types, some control figures have been retabulated for convenience in both this and in subsequent tables.

Table III. Carbohydrate diet

Exp.	Group	No. of animals	Liver fat p.c.	Weight of liver fat per mouse g.	D_2 in liver fat atoms p.c.	Av. D_2 in liver fat atoms p.c.	Weight of depot fat per mouse g.	D_2 in depot fat atoms p.c.	Av. D_2 in depot fat atoms p.c.	D_2O in body water p.c.
3	Controls fed 14 days on diet with 20 p.c. linseed oil containing 6·51 atoms p.c. deuterium	8	4·4	0·036	3·92	3·80	4·5	2·91	2·80	0·54
		7	4·3	0·037	3·67		5·3	2·69		0·43
	Carbohydrate diet 52 hr.	10	5·7	0·048	2·32	2·32	5·3	2·87, 2·87	2·87	—
	Carbohydrate diet 7 days	8	11·5	0·096	1·10, 1·12	1·15	3·5	2·38	2·46	0·07
		6	9·9	0·090	1·20		4·0	2·54		0·14
5	Controls fed 14 days on diet with 15 p.c. linseed oil containing 6·65 atoms p.c. deuterium	8	Lost	—	—	—	5·2	2·51	2·55	0·25
		7	„	—	—	—	4·8	2·58		0·23
	Carbohydrate diet 7 days	8	9·2	0·093	1·15	1·04	3·2	2·47	2·34	0·20
		7	8·4	0·085	0·92		3·5	2·20		0·18
6	Controls fed 14 days on diet with 20 p.c. linseed oil containing 6·69 atoms p.c. deuterium	8	5·6	0·074	3·10	3·11	3·7	2·46	2·51	0·30
		7	4·4	0·063	3·11		4·2	2·55		
	Carbohydrate diet 7 days	8	7·4	0·096	1·10	1·08	4·8	2·03	1·94	0·06
		7	6·9	0·093	1·05		3·5	1·85		
	Carbohydrate diet 7 days followed by 52 hr. fasting	7	13·8	0·174	1·49, 1·47	1·48	1·3	1·73	1·73	0·15
		7	15·9	0·214	1·98, 1·96	1·97	3·0	2·23	2·23	0·15

In Exp. 3 after 52 hr. on the carbohydrate diet there has been a slight increase in total liver fat. The deuterium content of the liver fat has dropped markedly below that of the liver fat of the control mice and is, furthermore, significantly less than that of the depot fat. After 7 days on the carbohydrate diet the total liver fat has doubled in amount, and its deuterium content has dropped to 1·15 atoms p.c. Only a slight drop in the deuterium content of the depot fat was observed.

In Exp. 5 the liver fat of the control animals was unfortunately lost. The remaining figures for liver fat and depot fat are, however, in excellent agreement with those found in Exp. 3. Similar results were obtained in Exp. 6, although here there was a somewhat larger drop in the deuterium content of the depot fat after 7 days on the carbohydrate diet.

The fact that the deuterium content of the liver fat of the animals fed 7 days on the carbohydrate diet was so far below that of the depots indicates strongly that the increase in fat came from a non-deuterium-containing source, that is, possibly from conversion of carbohydrate to fat. This conversion may have taken place entirely in the liver as little decrease in the deuterium content of depot fat occurred. The small drop in the level of depot deuterium is probably due to the storage of fat that has been formed from carbohydrate. Schoenheimer & Rittenberg [1936], on the other hand, found that the deuterium in the depot fat of mice fed a brown bread diet for 6 days almost entirely disappeared. In a later paper Rittenberg & Schoenheimer [1937] conclude that the rate of disappearance may be somewhat slower than they originally thought. Their results interpreted in the light of the present work suggest as one possibility the presence of some factor in their diets or experimental procedure which caused the transfer of large amounts of fat (presumably made from carbohydrate) from the liver to the depots.

The fact that 7 days' feeding of a carbohydrate diet to mice lowered the deuterium content of the liver fat far below that of the depots made it possible to check the conclusions drawn from the starvation experiments. Attention is drawn to the results shown in Table III, Exp. 6, obtained by starving a group of mice for 52 hr. after they had been maintained on the carbohydrate diet for 7 days. Starvation resulted in a marked increase in total liver fat; at the same time a decided increase in the deuterium content of the liver fat occurred. The increase was greatest in the group of animals which showed the higher level of deuterium in their depot fat. This experiment provides even more conclusive evidence that the increase in liver fat that occurs in mice following starvation results from a transport of fat from the depots to the liver.

In both Exp. 3 and Exp. 6 there was a marked fall in the percentage of deuterium oxide in the body water after maintenance of the animals for 7 days on the carbohydrate diet. This decrease was much less in Exp. 5. The decrease that occurred in Exp. 6 was followed by a significant rise when the animals were starved. The amount of deuterium oxide in the body water is an indication of the amount of deuterium-containing fat that is being oxidized. The results of determinations of this value

made after 7 days on the carbohydrate diet suggest that little depot fat is being burned. The marked increase that occurs on starvation suggests utilization of depot fat.

Protein feeding.

The small but significant decrease that occurred in the deuterium content of the depot fat of mice fed a carbohydrate diet could have been due to a breakdown and resynthesis of fat in the depots with a consequent loss of deuterium. This explanation is improbable in view of the fact that no change occurred in the deuterium content of the depot fat of mice that had been starved for 7 days (see Table II). The following experiments supply further evidence that the most probable explanation of the drop in deuterium percentage in depot fat which occurred in the mice when transferred to the carbohydrate diet is storage of fat formed from carbohydrate.

Groups of mice were fed a diet similar to that used in the carbohydrate feeding experiments with the exception that the 76 p.c. of sucrose was replaced by an equal quantity of meat powder. The results are shown in Table IV. After 7 days on this diet no change occurred in the deuterium

TABLE IV. Protein diet

Exp.	Group	No. of animals	Liver fat p.c.	Weight of liver fat per mouse g.	D_2 in liver fat atoms p.c.	Av. D_2 in liver fat atoms p.c.	Weight of depot fat per mouse g.	D_2 in depot fat atoms p.c.	Av. D_2 in depot fat atoms p.c.	D_2O in body water p.c.
6	Controls fed 14 days on diet with 20 p.c. linseed oil containing 0·69 atoms p.c. deuterium	8	5·6	0·074	3·10	3·11	3·7	2·46	2·51	0·30
		7	4·4	0·063	3·11		4·2	2·55		
	Protein diet 3 days	9	3·6	0·056	1·91, 1·89	1·90	3·8	2·45	2·45	0·15
	Protein diet 7 days	7	2·7	0·041	1·52	1·49	4·5	2·54	2·55	0·00
		7	2·6	0·040	1·46		3·3	2·56		
4	Controls fed 14 days on diet with 15 p.c. linseed oil containing 4·90 atoms p.c. deuterium	8	5·8	0·061	2·06	2·08	6·2	2·03	2·05	0·23
		7	5·0	0·049	2·10		5·7	2·06		0·18
	Protein diet 3 days	5	5·8	0·054	1·52	1·51	5·0	2·19	2·16	0·17
		5	4·7	0·046	1·49		4·8	2·13		
	Protein diet 14 days	8	4·1	0·044	0·89	0·79	3·6	1·86	1·88	0·00
		7	4·9	0·053	0·69		3·6	1·90		
	Protein diet 3 days with exposure to carbon tetrachloride	8	13·9	0·199	1·90	1·94	3·7	2·12	2·11	0·42
		7	14·7	0·257	1·97		4·2	2·10		0·33

content of the depot fat and it is doubtful if even after 14 days a significant decrease has taken place. Since both after a long period of starvation and after feeding a protein diet no drop occurred in the deuterium content of depot fat, it is concluded that the deuterium in the fat is stable under

these conditions in the animal's body, and further, that no breakdown and resynthesis of fat occur in the depots. In view of these considerations, storage of fat formed from carbohydrate appears to be the most logical explanation of any decrease occurring in the deuterium content of depot fat of mice fed a carbohydrate diet.

The deuterium content of the liver fat of the protein-fed mice shows a marked decrease. There is no significant increase in the total fat of the liver. Several explanations of this result are possible: (1) selective removal of deuterium from liver fat, (2) a difference between the deuterium content of different fractions of liver fat with removal of those of high deuterium content, (3) conversion of protein to fat, (4) saturation or desaturation of liver fat with consequent loss of deuterium, (5) breakdown of deuterium fat with resynthesis, resulting in a disappearance of deuterium.

Selective removal of deuterium from liver fat is improbable in view of the evidence available indicating that the animal does not distinguish between protium and deuterium.

To find if a difference between the deuterium content of different fractions of liver fat existed, the following experiment was carried out. Twenty rats were fed for 3 days on the usual diet containing 20 p.c. linseed oil (deuterium content of oil was 6·29 atoms p.c.). A separation of the depot and liver fat into phospholipin and neutral fat fractions was effected. Sinclair's [1930] method was employed. The experimental results are shown in Table V. Although there is a significant difference

TABLE V. Distribution of deuterium in phospholipin and neutral fat

20 rats fed for 3 days on diet containing 20 p.c. linseed oil
(D_2 content of linseed oil = 6·29 atoms p.c.)

	Total fatty acids	Neutral fat (fatty acid)	Phospholipin (fatty acid)
Livers	3·11, 3·18	2·59, 2·61	3·58, 3·52
Bodies	1·20, 1·19 1·16*	1·15 1·16*	1·24, 1·22 1·29*, 1·25*

* Duplicate saponifications.

between the deuterium content of the liver neutral fat and phospholipin fractions, it is not sufficiently great to account for the low deuterium value of liver fat obtained in the protein feeding experiments even if one considered it possible that all of the phospholipin could be selectively removed. In agreement with the results obtained using mice there was no significant difference in the deuterium content of the phospholipin and neutral fat fractions of the depots.

The present data are insufficient definitely to show which of the other explanations of the decrease in deuterium content of liver fat is correct. It is evident that no appreciable interchange between liver and depot fat was taking place since no change in the amount of deuterium in the depot fat occurred.

The fact that no detectable amount of deuterium was present in the body water even after 7 days on the protein diet suggests that little deuterium-containing fat was being oxidized. Protein must have supplied a large part of the energy for metabolic processes. On the other hand, the fact that a decrease in total depot fat had occurred after 14 days on the protein diet (see Table IV, Exp. 4) showed that some depot fat had been utilized during this period.

Exposure of mice to carbon tetrachloride vapour.

Following the initial period of feeding the diet which contained deuterium fat, two groups of mice were maintained for 3 days on the high-protein diet. One group was exposed for 6 hr. daily to 5200 parts per million of carbon tetrachloride vapour in an exposure chamber that has been described elsewhere [Barrett *et al.* 1938]. The second group served as controls. The results are shown in Table IV, Exp. 4. Exposure to carbon tetrachloride vapour has resulted in a tremendous accumulation of fat in the liver. The deuterium content of the liver fat in the exposed group was 1·94 atoms p.c. compared with 1·51 atoms p.c. in the unexposed animals. The increase in liver fat came therefore, in large part, from a deuterium-containing source, that is, from the depots.

Stability of the deuterium-containing fats.

In conclusion, some data are presented to indicate that no appreciable interchange between deuterium and protium was taking place in these experiments. Since fat transport and conversion were being studied, it was important to show that significant amounts of deuterium were not entering into combination with other constituents of the body. With regard to the first point, duplicate saponifications of the fat were carried out in many instances. As unstable deuterium interchanges with water and sodium hydroxide, the close agreement of the deuterium analyses in these experiments is excellent evidence that the deuterium being measured was stable.

Determinations were carried out on protein fractions separated from rat and mouse bodies and livers by precipitation with trichloracetic acid. After careful drying the protein was combusted. Due to the

presence of volatile oxides of nitrogen, the purification of the water resulting from combustion was modified in the following way. It was *intimately* mixed with about 0·05 g. of potassium hydroxide and transferred to a clean tube before the final distillation was carried out. No chromium trioxide was added to the sample, otherwise the procedure was the same as that used for the fats. Satisfactory purification was indicated by the results of a colorimetric *p*H determination.

Glycogen fractions were separated from the livers and depots of rats that had been fed for 4 days on a 20 p.c. fat diet (fat contained 6·69 atoms p.c. deuterium). The results of the analyses of these constituents are summarized in Table VI. The amount of deuterium in tissues other

TABLE VI. Distribution of deuterium in animal tissues

Animals	Percentage of deuterium-containing linseed oil in diet	D_2O in body water p.c.	Atoms p.c. deuterium in: Protein Body	Protein Liver	Fat Body	Fat Liver	Glycogen Body	Glycogen Liver
15 mice	15 p.c. for 9 days,* atoms p.c. D_2=5·90	0·10	0·00	—	0·92	1·24	—	—
15 mice	20 p.c. for 14 days, atoms p.c. D_2=6·20	0·42	0·06	—	2·25	3·51	—	—
20 rats	20 p.c. for 3 days, atoms p.c. D_2=6·29	0·06	0·01	0·05	1·20	3·17	—	—
10 rats	20 p.c. for 4 days, atoms p.c. D_2=6·69	0·10	—	—	2·62	2·86	0·00	0·04

* In this experiment the mice were fasted 48 hr. before being killed.

than fat is not more than a fraction of the deuterium content of the body water. This is in agreement with the findings of Smith *et al.* [1936]. These experiments show that the deuterium is a stable label when used for studies in fat metabolism. The remarkably constant level of deuterium maintained by depot fat in the animals which were starved and in those which were fed a protein diet appears to be conclusive evidence of the stability of deuterium-containing fats *in vivo*.

SUMMARY AND CONCLUSIONS

1. A method for analysing the deuterium content of solutions of deuterium oxide and water has been described. The method involves the use of a small pycnometer and a torsion microbalance of original design. The sensitivity of the balance was such that 6γ produced 1 mm. deflexion of the pointer. An analysis made on a sample of 0·3 g. is accurate to $\pm$0·03 p.c. deuterium oxide.

2. In confirmation of the results of previous investigators who have used other methods of labelling fatty acids the findings in these deuterium studies indicate that the principal, if not the only, source of the excess fat which accumulates in the liver during fasting is the body depots.

3. The results indicate that the fat which accumulates in the liver when certain extracts of the anterior pituitary gland are administered is also derived from this source.

4. When mice are exposed to carbon tetrachloride vapour the increase in liver fat apparently comes in large part, if not entirely, from the depots.

5. When fat containing deuterium is deposited in the body reserves and the animals are then placed on a diet low in protein and other lipotropic factors but rich in carbohydrate, the fat which accumulates in the liver is not derived from the depots. The most probable source of this fat is the carbohydrate of the diet.

6. When diets rich in protein are given to animals whose depot fat contains deuterium, there is no transfer of fat from depot to liver. Furthermore, the deuterium content of the depot fat remains surprisingly constant for periods up to 14 days. No final explanation can at present be given for the interesting decrease in deuterium content of liver fat under these conditions.

7. Further evidence of the stability of fats containing deuterium both *in vitro* and *in vivo* has been secured.

It is a pleasure to acknowledge the helpful co-operation of our colleague, Dr James Campbell, in certain of these experiments.

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J. Physiol. (1938) 93, 382-400 612.15

ON THE ACCURACY OF THE THERMOSTROMUHR METHOD FOR MEASURING BLOOD FLOW

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(*Received* 25 *May* 1938)

The thermostromuhr method for measuring blood flow has been fully described by Rein [1928, 1929*a*, *b*]. Its principles are as follows. High-frequency current is applied by small electrodes at opposite ends of a diameter of the unopened vessel in order to heat the streaming blood within. The temperature rise produced by the current is measured a short way downstream by a thermojunction pressing against the wall of the vessel. The rise in temperature is an inverse function of the blood flow. The measurement of the rise in temperature presents a difficulty because the temperature of the animal's blood may change. To get over this difficulty a second junction is placed upstream to the heated region and connected differentially (or in opposition) with the first (downstream one).

Some difficulties in our early experiments made us suspect that in spite of the use of differential junctions changes in the animal's blood temperature would affect the accuracy of the results. Small blood temperature changes seemed to affect the temperatures of the junctions unequally. The significance of the errors in the flow measurements seemed to depend on the size and rapidity of the blood temperature change and on the junction temperature difference set up by the heating current.

The object of this paper is to describe findings which show that, in some measurements, blood temperature changes may make the method unreliable.

We have not found very much in the literature about rapid changes in blood temperature. In the last century physiologists were interested in the amount of heat given out by different organs and in the conduction of this heat from place to place by the blood stream. Claude Bernard [1876] gives a detailed account of the "topography" of blood temperature in the great vessels. He shows that in animals the temperature of the

arterial blood is almost uniform in the great arteries of the trunk and limbs. On the other hand, the temperature of the venous blood in the jugular and femoral veins is about 1° C. below arterial blood temperature. The difference in the arterial and venous blood temperature depends on the temperature of the animal's surroundings. In man several observers, using thermoelectric methods, have found that venous temperature is low and depends on room temperature. Foged [1930] found that the temperature of the blood in the antecubital veins was, on the average, 2·6° C. lower than the rectal temperature. Harris & Marvin [1927] found that the temperature of the blood in the veins on the dorsum of the hand was between 34·3 and 35·8° C. Placing the hand in a water bath, the water being at 25·26° C., lowered the temperature of the venous blood 6·8° C. in 2 min. Wright & Johnson [1932] found that the temperature of the blood in the median cubital, jugular, and saphenous veins was, on the average, 1·2° F. below mouth temperature, the greatest observed difference was 13° F. Such observations, we think, show that changes in the skin circulation, brought about by nervous or chemical means, might cause quite large and rapid changes in venous blood temperature. A rather significant fact shown by Claude Bernard [1852] was that cutting the cervical sympathetic in dogs, cats and horses raised the temperature of the nose and ear passages 3–4° C. in a few minutes.

A rapid rise in blood temperature might be expected in muscular activity. We have not found any blood temperature measurements, but the inference may be drawn from the fact that muscle temperature rises rapidly during activity. Chauveau [1891] found that the temperature of the horses masseter rose 0·42° C. in 10 min. eating, and assumed that the temperature of the blood leaving it rose by the same amount. Béclard [1861] bound a thermometer to the upper arm with many turns of flannel. After about 2 hr. complete rest the temperature became constant to a fiftieth of a degree. A 5 kg. weight was then held for 5 min., the temperature rose 1·35° C. in 10 min. Béclard attributed the rise in temperature to heat production caused by contraction of the biceps. Becquerel & Breschet [1835] put a thermojunction into the fleshy part of the biceps. When the subject was sawing a piece of wood the temperature of the muscle rose 1·0° C. in 5 min. One of us put a thermojunction into the human gastrocnemius; the junction was 7 cm. from the surface. The temperature of the muscle rose 1·5° C. in 1½ min. standing running.

The temperature in the brain and liver has been measured by Crile & Rowland [1922*a*, *b*]. When a rabbit was under nitrous oxide ether

was given, the animal struggled and the temperature of its brain fell 2·2° C. in 2 min. Stimulation of the uncut sciatic nerve lowered the temperature of the liver 1·6° C. in half a minute. An unanæsthetized rabbit was given 500 μg. of adrenaline intravenously, a very large dose, and the temperature of its brain rose 1·1° C. in 2 min.

A general survey of this literature shows that some physiological and pharmacological procedures may cause blood temperature changes, especially venous blood temperature changes, amounting perhaps to a degree or more in less than 5 min.

Our results fall into three groups. First, thermostromuhr perfusion experiments. The temperature of the perfusion fluid was altered from time to time and the behaviour of the differential thermojunctions was recorded. These experiments show that changes in the temperature of the perfusion fluid of about 1° C. affected the temperatures of the junctions unequally. Secondly, thermostromuhr experiments on the animal, but without heating the vessel. These experiments show that the changes in the blood temperature caused by adrenaline were large enough to cause unequal heating of the junctions. Thirdly, some experimental work and a discussion on the significance of the errors likely to be found in the flow measurements.

Perfusion experiments

Method

Each thermoelement has to be calibrated in a perfusion experiment to find the relation between the blood flow and the deflexion of the galvanometer. The technique has been described by Rein [1928, 1929*a*, *b*] and we followed it as closely as possible. A few important details should be mentioned.

Unless otherwise stated, the type of element used was that in which the junctions were joined by a straight piece of constantan wire running along the bottom of the inner wall of the element. This type is referred to by Rein [1928] as "Form III". The important specifications were as follows. The distance between the junctions was at least 2½ times the internal diameter of the element. The distance from each junction to the edge of the element was at least 1 mm. The area of the heating plates in sq. mm. was equal to the internal diameter of the element in mm. The size of the wires forming the junctions was between 0·1 and 0·3 mm. according to the internal diameter of the element. (Rein [1928] used to use junctions of 0·03 mm. wire, he now uses sizes up to about 0·3 mm. [Smyth, 1937].) Each junction was covered with a thin layer of cellulose varnish. The thickness of the element wall varied with the

size of the element; for an element of 5 mm. internal diameter it was 1–2½ mm.

The thermocouple galvanometer was made by Downing. Its sensitivity varied according to the amount of magnetic shunting required for critical damping. The sensitivity was about $1{\cdot}2 \times 10^{-7}$ V., $5{\cdot}8 \times 10^{-9}$ A. per mm. at 1 m. Its internal resistance was 19 ohm. Its period was 1·2 sec. The short circuit zero was often checked and varied by less than 1 mm. at 1 m. The usual working distance was 4·5–5 m.

Mr Cowan showed us how to standardize the thermocouple circuit so that the deflexions could be converted into °C. junction temperature difference. This is not usually done, but it has great advantages which will be seen later. According to Hodgman [1933], from data in the *International Critical Tables*, 0·1° C. difference in the temperatures of two copper-constantan junctions sets up an effective e.m.f. of about 4×10^{-6} V. After each experiment 4×10^{-6} V. was put into the thermocouple circuit from a battery and potential divider; this deflexion thus corresponded to about 0·1° C. junction temperature difference. Strictly speaking each couple should have been calibrated separately to get the exact relation between junction temperature difference and e.m.f. This was not done. For our purpose it was sufficient to assume the "ideal" relations between junction temperature difference and e.m.f.

The vessel was perfused by gravity from a 10 l. bottle. The flow was regulated by a screw clip on the tubing leading from the perfusion bottle. It was measured by a stop-watch and measuring cylinder. Except when stated, defibrinated ox blood was used; its temperature was carefully brought to within 0·5° C. of room temperature before the experiment was begun (see later).

Carefully selected pieces of calf, goat, sheep or dog vein were chosen, those with valves or branches were avoided. Special flanged cannulæ were used to avoid irregularities on the inner surface of the perfusion system. Great care was taken to fit a suitable sized element, of slightly smaller diameter than the vessel, nicely round the vein and to fix the leads so that it could not slip. The top of the element and vein were painted with collodion. The thermos flask and vessel were horizontal; the air in the thermos was kept moist by a small piece of wet cotton wool. The height of the outflow tube was adjusted so as to keep the vein under a positive pressure of a few centimetres of water.

A second thermocouple system was used to show changes in the temperature of the perfusion fluid. In some cases one junction was in the air of the thermos flask and the other in the blood a short distance

upstream from the element. In others the junctions lay, one on the outer and the other on the inner side of the element wall. The junctions were standardized by the introduction of 4×10^{-6} V. as described above.

In the experiments to be described the heating current was not used and the batteries of the high-frequency generator were disconnected to avoid any escape of stray currents into the thermocouple circuit.

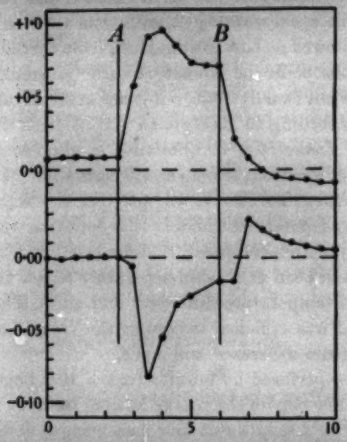

Fig. 1. Blood perfusion of dog's jugular vein. 3 mm. element (*R*). Flow 53 c.c./min. Ordinates: Upper curve, difference in temperature between blood and thermos air in °C. The readings are + when the blood was warmer than the thermos air. Lower curve, junction temperature difference in °C. The readings are + when the downstream junction was warmer than the upstream. Abscissæ: Time in minutes. Lint moistened with warm water was wrapped round the tubing connecting the perfusion bottle to the vein during the interval *A* to *B*.

No readings were taken till perfusion had gone on long enough for the temperature of the apparatus to settle down.

The symbols (*R*) and (*L*) are used in the legends below the figures to show whether the element was put on to the vessel with the leads to the right or to the left side of a person looking downstream.

Results

The following perfusion experiments were done to find out how the differential junctions were affected by rapid changes in the temperature of the blood.

In one series of experiments the temperature of the blood was raised about 1° C. by wrapping a piece of lint moistened in warm water round the tube taking blood from the perfusion bottle to the vein. Readings of the junction temperature difference were taken at minute or half-minute intervals both before and after warming the blood had begun. Fig. 1 is

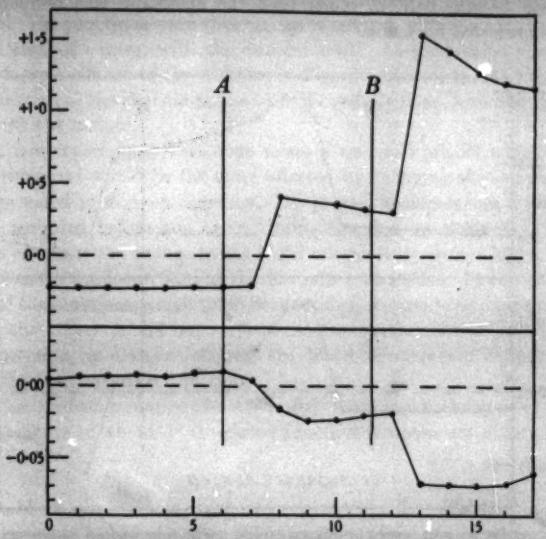

Fig. 2. Blood perfusion of dog's jugular vein. 4 mm. element (*L*). Flow 50 c.c./min. Ordinates: Upper curve, difference in temperature of the outside and inside of the element wall in °C. The readings are + when the inside was warmer than the outside. Lower curve, junction temperature difference in °C. The readings are + when the downstream junction was warmer than the upstream. Abscissæ: Time in minutes. Blood above room temperature was added to that in the perfusion bottle at *A* and again at *B*.

typical of the results with 3, 3·5 and 4 mm. elements. The upper curve shows that the warmed lint applied during time *A* to *B* raised the temperature of the blood entering the vein by about 0·8° C. The lower curve shows that the temperatures of the two junctions were not equally affected, either during or immediately after the procedure. One and a half minutes after putting the lint on at *A*, the downstream junction was 0·08° C. cooler than the upstream.

In other experiments the temperature of the blood was altered by adding warmed or cooled blood to the blood in the perfusion bottle. An example from the results with 4, 5, 6 and 7 mm. elements is seen in Fig. 2. Warmed blood was added at *A* and again at *B*. The temperature of the blood entering the vein was raised in two steps by 1·5° C. The downstream junction became 0·07° C. cooler than the upstream, this temperature difference lasted for 4 min.

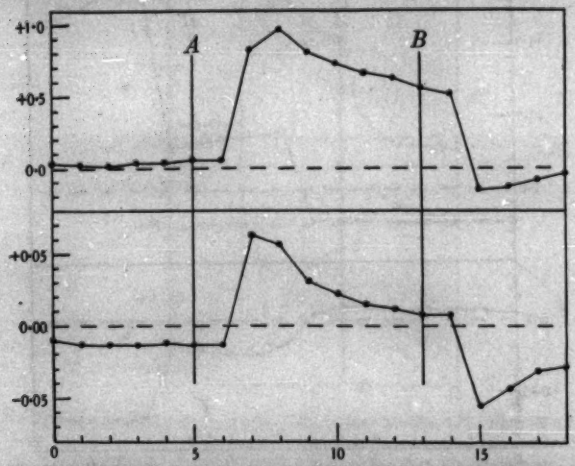

Fig. 3. Saline perfusion of 3 mm. element. No vein. Bare junctions. Ordinates and abscissæ as in Fig. 2. Saline above room temperature was added to that in the perfusion bottle at *A*, saline below room temperature was added at *B*.

We thought the difference in the behaviour of the junctions might be due to differences in the thicknesses of their insulation. Each junction was covered with a thin coat of cellulose varnish. It seemed that elements with bare junctions, sometimes used by Rein *et al.* [1935], might be less unsteady. A 4 mm. element with bare junctions was made and tested. The results were very little better than those obtained with the ordinary elements. (Becquerel & Breschet [1835], Béclard [1861] and Grützner & Heidenhain [1878] found that temperature measurements of the order of 10^{-2} °C. were not reliable if one of the junctions of a couple was in contact with an animal tissue or fluid.)

Although Rein [1931] found that the method gave good results as long as the element was fitted to the vessel with reasonable care we wished to make quite sure that our results were not due to improper application of the element. This was done by eliminating the vein altogether. The top of the element was bridged over with plastic material, and the tube so formed was carefully joined to the perfusion tubing in place of the blood vessel. The junctions were then in direct contact with the blood stream (and also, of course, with the element wall). As the results, shown in Fig. 3, were similar to those shown in Figs. 1 and 2 we thought that the application of the element to the vein, if carefully done, had very little to do with the matter.

A few experiments were done to see if we could predict which of the two junctions would be the more affected by changing the temperature of the blood in a given direction. The junction temperature differences were recorded before and after turning the element through 180°. In three elements the change in the blood temperature always had a bigger effect on the upstream than on the downstream junction. In two elements one of the junctions, which could be identified, seemed to be more sensitive than the other. It did not seem to matter whether it was upstream or downstream, it always followed the blood temperature change more quickly than the other junction.

The perfusion experiments showed that rapid changes of blood temperature of about 1° C. caused junction temperature differences of about 0·07° C.

Animal experiments

In these experiments the behaviour of the differential thermocouple was recorded before and after the intravenous injection of adrenaline in saline. This procedure seemed particularly suitable for the following reasons. Injections of hormones and drugs affecting the circulation are made in many physiological and pharmacological experiments. It is a typical experimental procedure and so is suitable for testing the accuracy of a general method for measuring blood flow. Adrenaline itself is made in the body and is poured into the blood in amounts which vary with the condition of the animal. It seemed likely that adrenaline would affect blood temperature. It seemed likely that it might increase blood temperature, hot blood from the liver and spleen might be added and the circulation through the cold skin might be cut down. It also had the great advantage that the necessary operative interference was very slight, and the animal could not suffer from shock.

Method

The experiments were done under typical conditions. Two kinds of animal, three goats and one dog, were used under chloralose anæsthesia. The operating table had a copper bath filled with warm water. There was an electric stove about 10 ft. from the animal.

The blood vessel to which the element was applied was in the living animal and not in the thermos, otherwise the thermostromuhr technique was as that used in the perfusion experiments. The vessel was prepared and the element carefully put on as directed by Rein [1929*a*]. It was fixed in position with collodion. The edges of the wound were carefully closed, and before any readings were taken sufficient time was allowed for the temperature conditions in the depths of the wound to become normal.

With goats 2 and 3 and with the dog the wound was covered with a thick sheet of cotton wool. The outer surface of the wool was gently warmed by the heat from an electric light bulb. Also the leads to the element were fixed in a clamp some inches from the wound to prevent displacement of the element by accidental movement of the wires.

In these experiments elements were fitted to the vessels, altogether, eleven times. The junction temperature difference was never steadier or smaller than in the perfusion experiments. This is not in keeping with Rein's [1929*b*] finding that temperature conditions in the animal are more uniform than in the thermos. The discrepancy may be due to: (1) The very carefully controlled temperature conditions in our perfusion experiments. Perfusion was started before the thermos was put over the vein. The second thermocouple system then showed the temperature of the perfusion fluid relative to the room air. Warmed or cooled blood was added to that already in the perfusion bottle till the blood flowing into the vein was within less than 0·5° C. of room temperature. The thermos flask was then placed over the vein. Figs. 1, 2 and 3 show that the initial temperature differences between the blood and the thermos air were only 0·1, 0·25 and 0·05° C. respectively. If the blood temperature had been 2 or 3° different from that of the room air, as in Rein's perfusion experiments [1928], the junction temperature differences might have been much greater and less steady. (2) In the dog experiment the element was fitted to the femoral vein. The femoral artery probably touched the element at some point. The temperature of the blood in the two vessels may have been different by several degrees, there may have been a large temperature gradient across the part of the element wall separating the two vessels.

We do not know whether the junctions lay in or near this temperature gradient. As Mr Cowan has pointed out, the position of the junctions relative to the artery probably would have affected the junction temperature difference.

A burette and cannula was attached to the central end of the jugular vein in all experiments. With goats 1 and 2 the adrenaline was injected by hypodermic syringe into the rubber tubing joining the burette to the cannula and was washed into the animal by 5 c.c. of saline from the burette. This had the possible disadvantage that the temperature of the saline was sometimes rather low. With goat 3 and with the dog the adrenaline and the saline were kept warm in the water bath. The necessary amounts, 5 c.c. in all, were drawn into the syringe which was then put back into the water bath. When necessary the syringe was taken out and the adrenaline solution was squirted into the rubber tubing between the screw clip and the cannula, the screw clip was not opened.

In the animals in which the element lay on the carotid the jugular of the opposite side was used for the adrenaline injections. The slight movements of the tubing and cannula caused by inserting the syringe needle had no effect on the junction temperature difference.

Results

The following results are typical of the behaviour of the differential junctions after adrenaline injections.

Fig. 4 was drawn from the observations made on goat 1. The element lay on the carotid artery. Between curves 1 and 2 the element was taken off and carefully replaced. Between curves 2 and 3 the element was taken off, turned through 180°, and replaced. Between curves 3 and 4 the element was taken off again, turned back through 180° to its original position and replaced. Before readings were taken enough time was allowed for the temperature conditions in the wound to become normal. The readings in curve 1 were taken after the element had been in position for over an hour. The adrenaline and saline injections, at *A*, were all followed by changes in the junction temperature difference. In two cases the change amounted to 0·04° C., in the other cases it was less.

Fig. 5 was drawn from the experiment on the dog. The blood pressure at the beginning of curve 1 was 150 mm. Hg. The element was on the femoral vein. In addition to the ordinary differential couple the element had a second couple system with junctions on the inner and outer surfaces of the wall. The upper curves show the temperature difference between the inside and outside of the element. The lower curves show

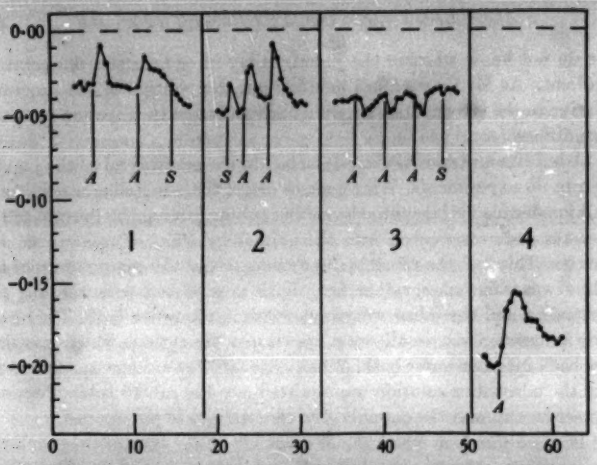

Fig. 4. Goat's right carotid artery. 3 mm. element. The orientation was (*R*), (*R*), (*L*), (*R*) for curves 1, 2, 3 and 4 respectively. Ordinates: junction temperature difference in °C. The readings are + when the downstream junction was warmer than the upstream. Abscissæ: Time in minutes. 5 c.c. intravenous injections of adrenaline in saline at *A*. Reading from left to right the doses were 5, 5, 5, 10, 5, 10, 5, 100μg. Control injections of 5 c.c. saline at *S*.

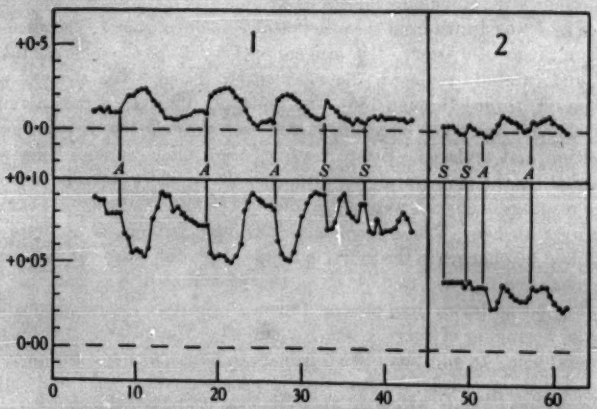

Fig. 5. Dog's right femoral vein. 3 mm. element (*L*). Ordinates and abscissæ as in Fig. 2. Intravenous injections of 50μg. adrenaline in saline at *A*. Control injections of saline only at *S*.

the temperature difference between the ordinary junctions of the element. There was an interval between curves 1 and 2 but the wound was not opened. The adrenaline injections, at *A*, caused the inner wall of the element to become relatively hotter than the outer, this showed a rise in blood temperature. In the first three injections the difference was 0·15° C. (upper curves). All adrenaline injections except the last changed the junction temperature difference (lower curves). In the first three injections the downstream junction became, relatively to the upstream, 0·04° C. cooler.

Control injections of saline only, at *S*, are shown in both Figs. 4 and 5. In four out of seven cases they had no effect. In three cases they had an effect which was probably because some adrenaline remaining in the stump of the jugular vein was washed into the circulation.

Sollmann & Gilbert [1938] and others have shown that adrenaline constricts the walls of large arteries. In our experiments adrenaline may have caused trivial movements of the vessel wall near the junctions. This may have had some effect on the junction temperature difference.

These experiments show that a common physiological procedure like the injection of adrenaline in saline, done under quite usual experimental conditions, may change the junction temperature difference by as much as 0·04° C.

The effect of rapid changes in blood temperature on the accuracy of the method

The errors in the flow measurements caused by blood temperature changes can be expressed graphically in a way which readily shows the relation between the various factors concerned.

Rein [1928, 1929 *a*, *b*] has shown that the galvanometer deflexion, G, and the blood flow, V, are related by the expression $GV^{\alpha}=K$, where K is a constant depending on the heating intensity, α is a constant which is less than 1 for most elements. It is never greater than 1. Since the galvanometer deflexion, G, is directly proportional to the junction temperature difference, T, we may write $TV^{\alpha}=k$, and with constant heating intensity $T_{\mathrm{I}}V_{\mathrm{I}}{}^{\alpha}=T_{\mathrm{II}}V_{\mathrm{II}}{}^{\alpha}$, where T_{I} and T_{II} are junction temperature differences set up by any two flows V_{I} and V_{II} respectively.

Imagine that at a given moment the actual flow is V_a with junction temperature difference T_a. A change in blood temperature then occurs which alters the temperature of the downstream junction relative to the upstream by t° C. Assuming the actual flow V_a to remain unchanged the junction temperature difference would become T_a+t. The flow would

appear to change but actually no change occurred. If V_0 is the apparent flow corresponding to the deflexion T_a+t, then

$$T_a V_a^{\alpha} = (T_a+t)\, V_0^{\alpha}$$

and

$$V_0 = \left(\frac{T_a}{T_a+t}\right)^{1/\alpha} V_a. \qquad \text{.....(1)}$$

If the blood temperature change warmed the downstream junction the values of t are $+$, the deflexion is increased and the apparent flow V_0 will be less than the actual flow V_a.

Now

$$\text{p.c. error in } V_a = \frac{V_a - V_0}{V_a} \times 100.$$

Substituting the value for V_0 found in equation (1),

$$\text{p.c. error in } V_a = 100\left[1-\left(\frac{T_a}{T_a+t}\right)^{1/\alpha}\right]. \qquad \text{.....(2)}$$

If the blood temperature change cooled the downstream junction relative to the upstream, the values of t are negative, the deflexion is diminished and the apparent flow V_0 will be greater than the actual flow V_a. The expression then becomes,

$$\text{p.c. error in } V_a = 100\left[\left(\frac{T_a}{T_a+t}\right)^{1/\alpha}-1\right]. \qquad \text{.....(3)}$$

Inspections of equations (2) and (3) shows that for any given value of α (for any given element) the percentage error in V_a depends on two factors: on T_a the junction temperature difference set up by the heating current, and on t, the junction temperature difference set up by the blood temperature change.

Equations (2) and (3) also show that with any given values of T_a and t the percentage error in V_a is an inverse function of α. The percentage error will be least with elements having an α of 1.

To draw the graph it is necessary to know the limits of T_a and t. The largest values of t in the perfusion experiments were 0·07° C., in the animal experiments they were 0·04° C.; 0 to $\pm$0·07° C. would cover our experiments, and will be taken as the limits, but other experiments and types of element may need different limits.

As will soon be seen, the values of T_a, the junction temperature difference set up by the heating current, are of very great importance to the significance of the percentage error. Unfortunately the ranges of T_a used in thermostromuhr experiments are usually not stated. Indeed Rein [1928, 1929*a*, *b*] and Baldes & Herrick [1931, 1937] seem to hold quite different views about the temperature difference generated between the upstream and downstream junctions by the heating current. To discuss this here would be to risk losing the thread of the present

argument. It is safe to say that both Rein and Baldes would probably agree that the junction temperature difference set up by the heating current could not be more than 1° C. For the moment, the limits of T_a will be taken as 0–1·0° C.

Fig. 6 was drawn from equations (2) and (3) taking the limits of T_a and t given above, and taking α as 1.

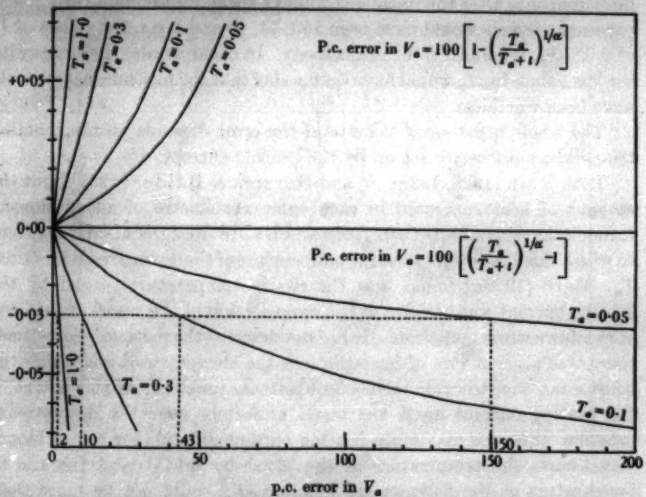

Fig. 6. V_a, actual flow. T_a, junction temperature difference caused by the heating current. t, junction temperature difference caused by change in blood temperature. α, a constant for each element. For further explanation see text.

The values of t were plotted as ordinates, and the percentage error in the flow measurements (in V_a) as abscissæ. The upper curve shows the percentage error when the change in blood temperature causes a rise in the temperature of the downstream junction, the values of t are $+$, the apparent flow is greater than the actual flow. The lower curve gives the percentage error when the change in blood temperature causes a fall in the temperature of the downstream junction, the values of t are $-$, the apparent flow is less than the actual flow.

The graphs show the great significance of T_a, the junction temperature difference set up by the heating current; the percentage error is very large with small values of T_a.

The importance of the T_a values is emphasized by taking a figure for t from the adrenaline experiments. In Fig. 5 the changes in blood temperature which followed the first three adrenaline injections changed the temperature of the downstream junction, relatively to the upstream, by about $-0{\cdot}03°$ C. Referring to Fig. 6 and taking the ordinate t is equal to $-0{\cdot}03°$ C. on the lower set of curves the percentage error in the flow measurements after the injections would depend on the value of T_a. The percentage errors would have been 150, 43, 10 and 2 p.c. for values of T_a of 0·05, 0·1, 0·3 and 1·0° C. respectively. In other words, the errors with the low values for T_a would have been so big that the measurements would have been worthless.

The whole question of the size of the error depends on the junction temperature difference set up by the heating current.

Both Rein [1928, 1929*a*, *b*] and Herrick & Baldes [1931] limit the amount of heat generated in each cubic centimetre of blood flowing through the element to 0·1 cal. Rein and Baldes disagree about the extent to which this heat would raise the temperature of the downstream junction, T_a. Rein [1929*a*] found that the rise in temperature caused by the heating current was uniform over a cross-section of the vessel at the level of the downstream junction. He did not describe the method. Sometimes there was a slight loss of heat through the element wall and along the constantan wire towards the cooler upstream junction. If this is true, if the heating current heats the blood uniformly near the downstream junction, then the maximum heating current, 0·1 cal. per c.c. of blood, would raise the temperature of the blood by 0·1° C. and the rise in temperature at the downstream junction, T_a, could not be more than 0·1° C. Fig. 6 shows that if the values of T_a are 0·1° C. or less changes in blood temperature may cause very large errors in the flow measurements.

Herrick & Baldes [1931] do not agree that the blood is uniformly heated. They say: "The fact that the galvanometer always records a temperature difference along the vessel wall many times greater than can exist in uniformly heated blood fails to lend support to the theory." If this is right the junction temperature difference, T_a, due to passing 0·1 cal. into each cubic centimetre of blood, must be many times more than 0·1° C. Fig. 6 shows then when the values of T_a are much more than 0·1° C. the errors due to blood temperature changes are much smaller.

We are very much indebted to Mr Cowan for a suggestion which enabled us to test these views.

We had done over sixty perfusion experiments to calibrate our elements. We had a series of readings for each element. For each reading

the corresponding values of 1, 2 and 3 given below were known and the values of 4 and of 5 were calculated from them:

1. The flow.
2. The junction temperature difference T_a.
3. The heating intensity.
4. The rise in the temperature of the blood assuming it to have been uniformly heated. Obtained from 1 and 3.
5. X, the ratio of 2 to 4.

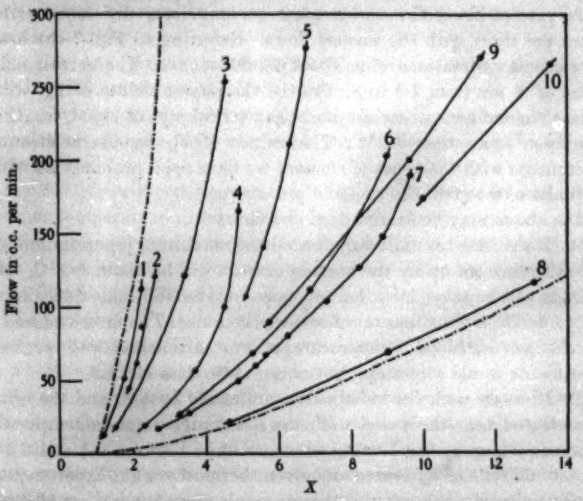

Fig. 7. The sizes and types of element were as follows: Curves 1, 6 and 7, 3·5 mm.; 8 and 10, 4·5 mm.; 9, 5·5 mm.; 3, 3·5 mm., no vein; 4, 3 mm., Baldes A.C.; 2, 4 mm., Baldes A.C.; 5, 5 mm., Baldes A.C. For further explanation see text.

The ratio X showed how much hotter the downstream junction was than it should have been on the assumption that the blood had been uniformly heated. When the ratio was 1 the blood could have been uniformly heated.

Fig. 7 shows the results. The flow is plotted against the ratio X. Each of the curves was drawn from the readings of a different calibration experiment. The sizes and types of element used are given below the figure.

All the curves lie inside the dotted lines. As the flows get smaller the limits converge and approach the value $X=1$. Rein's finding, that the blood is uniformly heated, may be nearly true for very low flows, lower than those we used. For the normal range of flows the values of X are greater than 1. For flows of 100 c.c./min. the vessel wall at the downstream junction was 2–12 times hotter than it should have been on the assumption that the blood was uniformly heated. For the range of flows we used in our calibration experiments our results support Baldes.

As the rise in the temperature of the vessel wall is an inverse function of the rate of blood flow the greatest galvanometer deflexions and T_a values are those with the slowest flows. Referring to Fig. 7 the lowest flows in our calibrations were about 25–50 c.c./min. The corresponding values of X are from 1·5 to 8. That is, the largest values of T_a, with a heating current generating not more than 0·1 cal./c.c. of blood, would not have been more than 0·8° C. The values of T_a for thermostromuhr experiments with the types of element we have used probably lie within the limits of 0 to 0·8° C.

The above may be summarized as follows:

(1) If the blood is uniformly heated the maximum junction temperature difference set up by the heating current will be about 0·1° C. Most readings will be taken with half or three-quarter full scale deflexions of 0·05 to 0·075° C. junction temperature difference. The errors caused by the changes in blood temperature in our adrenaline and perfusion experiments would sometimes have been more than 100 p.c.

(2) If, as we find, the blood is not uniformly heated, and the heat is concentrated near the vessel wall, the maximum junction temperature difference set up by the heating current will be between 0·1 and 0·8° C. The actual limits of T_a used in any given thermostromuhr experiment are usually unknown because the thermocouple circuit is not usually standardized and the galvanometer deflexions cannot be converted into degrees centigrade. The errors for our experiments would usually have been less than 100 p.c.

Discussion

Kramer [1936] tested the accuracy of the thermostromuhr. In one series of experiments he measured the percentage change in the flow in the femoral artery with the thermostromuhr and by a photometric method. Both methods were used simultaneously. Both showed that when the animal breathed 3·95 p.c. oxygen the flow increased by about 80 p.c. Both gave the same time relations for the changes in flow.

In other experiments Kramer measured the flow in the femoral artery by the photometric method only. Intravenous injection of 10 μg. of adrenaline caused changes in flow similar to those Rein had found with the thermostromuhr.

These experiments prove the accuracy of the thermostromuhr in the above experiments.

We do not think that this is a proof for thermostromuhr experiments in general, because:

(1) Other physiological and pharmacological procedures may cause much greater changes in blood temperature. For example, perhaps muscular activity.

(2) For any given procedure the blood temperature changes are probably different in arteries and veins.

(3) For any given procedure, and any given blood vessel, the blood temperature changes may vary with the temperature of the animal's surroundings.

(4) For given blood temperature changes the errors depend on the junction temperature difference set up by the heating current. If the junction temperature difference is less than 0·1° C. the errors will be very much greater than if the junction temperature difference is, say, 0·5° C.

(5) The Rein "Form III", Baldes A.C. [1933], Baldes D.C. [1937] and Schmidt & Walker [1935] elements are so different that a given blood temperature change will probably set up different junction temperature differences in these elements.

We believe that all thermostromuhr measurements should be carefully controlled to avoid errors due to blood temperature changes. Such errors are not likely to arise so long as the circulation is in a comparatively steady state. They are more likely to occur when rapid vascular changes are going on, when there is a quick redistribution of the blood, and when parts of the body are undergoing rapid changes in temperature due to activity or changes in surrounding temperature.

It would be quite easy to get an idea of the error likely to affect the flow measurements in an experiment by:

(1) Standardization of the thermocouple circuit so that the galvanometer deflexions could be turned into degrees centigrade junction temperature difference. The junction temperature difference T_a set up by the heating current would then be known.

(2) Doing the experiment a number of times, without the heating current, to find the limits of t, the junction temperature difference set up by the blood temperature changes.

(3) Reference to graphs, like Fig. 6, to find the percentage error corresponding to the values of T_a and t.

A statement of the values of T_a and t might be useful to others doing the same kind of experiment.

Summary

1. Physiological changes in blood temperature may affect the accuracy of the thermostromuhr method.

2. The significance of the error depends on several factors and especially on the junction temperature difference caused by the heating current.

3. The error in measuring the flow through the femoral vein after adrenaline would have been approximately 150, 43, 10 and 2 p.c. for junction temperature differences of 0·05, 0·1, 0·3 and 1·0° C. respectively.

4. A method is given for finding the significance of the error.

The authors collaborated in the earlier and most difficult stages of the work. W. M. Loughridge was unfortunately obliged to leave before the animal experiments were done.

It is a pleasure to acknowledge our gratitude to Mr S. L. Cowan for his very valuable advice about the thermostromuhr.

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EXPERIMENTS ON THE RELATION BETWEEN THE THYROID GLAND AND LACTATION IN THE RAT

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(*Received* 10 *June* 1938)

THERE has hitherto been a tendency to regard the role of the thyroid gland in lactation as a minor one [Nelson, 1936; Nelson & Tobin, 1937]. Nevertheless, in view of the importance of the thyroid as a regulator of general body metabolism it is to be expected that its secretion would exercise an important influence on a physiological function such as lactation, which involves a comparatively heavy strain on the metabolism. It is easy to envisage the thyroid affecting lactation both directly by regulating the metabolism of the mammary gland cells and indirectly by governing to some degree the level of milk precursors in the blood [Jones, 1935].

Certain experimental facts can be cited in support of this view. Stimulation of established lactation in the cow has been shown to result from the feeding of dried thyroid gland [Graham, 1934*a*; Herman *et al.* 1938], the injection of thyroxine [Graham, 1934*b*; Folley & White, 1936; Herman *et al.* 1938] and the injection of anterior lobe thyrotropic extracts [Folley & Young, 1938]. Contrary results obtained by de Fremery [1936] with the goat were probably due to overdosage with thyroxine. Further, Weichert & Boyd [1934] have shown that mammary development is more rapid, and the secretory phase earlier established, in pregnant rats receiving dried thyroid orally than in controls.

Unfortunately, experiments on the effect of thyroidectomy upon lactation have given results which disagree. Diminished milk secretion following thyroidectomy in the goat was observed by Grimmer [1918] and Grimmer & Paul [1930]. Graham [1934*a*], on the other hand, was hardly able to distinguish the effects of thyroidectomy on the milk

yield of a lactating cow from those of a control operation. The observations of Dragstedt *et al.* [1924], who found that thyro-parathyroidectomized bitches could rear their litters provided tetany was prevented, suggest that lactation is possible in the absence of the thyroid. The results most difficult to reconcile with the known facts regarding stimulation of lactation by administration of thyroid hormone are those of Nelson & Tobin [1937], who found that the average weaning weights of the young of rats thyroidectomized during pregnancy were very little, if at all, less than those of the young of control rats. Though these results seem to suggest that lactation in the rat is unaffected by thyroidectomy this interpretation would seem improbable in view of the known facts regarding the effect of the thyroid hormone on lactation. In this connexion it may be significant that the average weight (26·1 g.) of the young of "normal" rats at the 21st day from birth given by Nelson & Tobin is very much lower than the figure usually accepted as normal, namely, 40–45 g. Nelson & Tobin do not describe the type of rat used by them. If, however, it was one of those usually used for experimental work it would seem that even in their control rats lactation was subnormal.

In view, therefore, of the lack of agreement between the results of experiments on the administration of thyroid hormone to lactating animals on the one hand and of experiments on the effect of thyroidectomy on lactation on the other, it was considered worth while re-examining in rats the effect of thyroidectomy on lactation.

Experimental

Experimental animals and procedure. The animals used were hooded Norway rats from the colony maintained in this Institute by Dr S. K. Kon to whom I am indebted for facilities for carrying out this work. They were fed on the following diet *ad lib.*:

	p.c.
Whole wheat	69
Linseed cake	14
Casein (crude)	6
Dried brewers' yeast	5
Butter	5
$CaCO_3$	0·5
NaCl	0·5

In addition, each received as much fresh milk as she could drink, one slice of raw carrot daily and fresh raw liver three times weekly. It will be noticed that this diet provided adequate supplies of calcium.

The method of studying lactation employing as criterion the average growth rates of the sucklings has been described previously [Folley & Kon,* 1937]. As before, litters larger than eight were reduced to that number immediately after parturition, an even balance between males and females being maintained where possible. Rats with litters smaller than eight were distributed among the experimental and control groups so as to make the groups as nearly comparable as possible.

Lactating rats were thyroidectomized on the 6th day of lactation under avertin anæsthesia (1 c.c. 2½ p.c. avertin per 100 g. body weight intraperitoneally). Parathyroid tissue embedded in the thyroid was removed with it, but since no attempt was made to extirpate any parathyroid tissue not closely associated with the thyroids the operation is referred to in this paper, not as thyro-parathyroidectomy, but simply as thyroidectomy. Care was always taken not to injure the recurrent laryngeal nerve during the operation. In cases where the latter was accidentally injured the rats quickly developed stridor and invariably died within 3 days of the operation. The thyroid vessels were not tied off before the glands were removed, hæmostasis being secured by pinching the main vessels with forceps. In spite of this, considerable bleeding usually occurred during the operation. In a second group of lactating rats bleeding was largely prevented by using an electrocautery. Nevertheless, the recovery rate was not so good as with the first technique, a fair percentage dying because of injury to the trachea. A third group of lactating mothers were unilaterally thyroidectomized, as far as possible an approximately equal loss of blood to that involved in bilateral thyroidectomy being permitted. For controls, lactating rats were anæsthetized and the thyroids exposed but left intact.

The effect of thyroidectomy during lactation. The average growth curves for the litters of groups of six rats which underwent thyroidectomy and the control operation respectively on the 6th day of lactation are shown in Fig. 1.

It will be seen that the control operation caused a temporary check in the growth rate of the sucklings which thereafter grew steadily. On the other hand, lactation was, to say the least, seriously interfered with by thyroidectomy, since for 9 days after the operation the young of the thyroidectomized rats showed no weight increase and only began growing again on the 16th day of lactation. Since only one death occurred during the 9-day period following thyroidectomy when the average litter weight remained about constant, it is clear that after the operation the mothers must have been producing a small quantity of milk. The increase in the

average growth rate which began at the 16th day of lactation may be attributed to two causes: (1) the weaker members of the litters, which

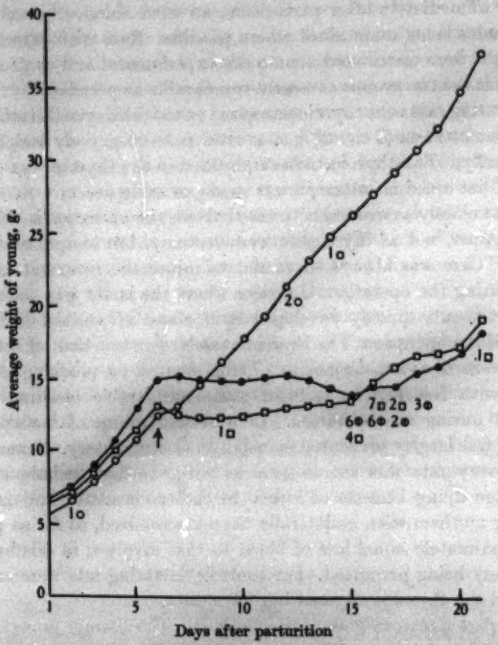

Fig. 1. Average growth curves of pooled litters. □—□ Group of six rats thyroidectomized on the 6th day of lactation. Number of pups at beginning of experiment = 44 (19♂♂, 25♀♀). ◑—◑ Group of four rats thyroidectomized on the 6th day of lactation and thereafter treated with thyroxine and parathormone (see text). Number of pups at beginning of experiment = 26 (12♂♂, 14♀♀). ○—○ Group of six rats which underwent exposure of the thyroids on the 6th day of lactation. Number of pups at beginning of experiment = 44 (18♂♂, 26♀♀). ↑ indicates the last observation before the operation. The numbers underneath the curves refer to the number of pups, belonging to the group indicated by the subscript, which were found dead on the relevant occasion of weighing.

were lightest in body weight, began to die of inanition on that day, and (2) the eyes of the strongest and heaviest pups were open by that time and they therefore began to have access to their mothers' food.

The lactation performance of thyroidectomized rats receiving injections of thyroxine was also studied. Four rats were thyroidectomized on the 6th day of lactation and thereafter given subcutaneous injections of 0·1 mg. thyroxine-sodium (B.D.H. Ltd.) dissolved in 0·5 c.c. water, daily until weaning, save that no thyroxine was injected on the 9th and 10th days of lactation, while double the usual injection was given on the 13th day of lactation. The least possible amount of NaOH was used for dissolving the thyroxine and the solution neutralized, to the point of reprecipitation, by addition of HCl.

In no case, either in this group or in the one previously described, were any obvious symptoms of tetany observed following thyroidectomy on the 6th day of lactation. Nevertheless, since thyroxine administration did not appear to have effected any improvement in the lactation performance by the 12th day of lactation, each rat in this group was given a subcutaneous injection of one unit of parathormone (Lilly) on the 12th and 15th days of lactation, in case the diminution in lactation following the operation was due to parathyroid deficiency. The average growth curve for the young of this group is also shown in Fig. 1 and it is evident that the thyroidectomized rats receiving the above treatment lactated no better than the untreated thyroidectomized rats.

Replacement therapy was attempted with a second group of three thyroidectomized rats. These were thyroidectomized as before on the 6th day of lactation, but hæmorrhage during the operation was minimized by use of an electrocautery. Immediately after the operation and every 4th day thereafter until weaning, or until the mother was removed from the experiment because of the death of all her young, each rat was given an intramuscular injection of 1 c.c. of solution containing 0·25 mg. thyroxine-sodium followed by a subcutaneous injection of one unit of parathormone. The average growth curve for the litters of these rats is given in Fig. 2 together with that for the group which underwent the control operation.

From the growth curve and from the number of deaths among members of the litters subsequent to the operations it is clear that this treatment effected no improvement in lactation as compared with untreated thyroidectomized rats.

Reproduction and lactation after thyroidectomy. Eight of the ten rats which had been thyroidectomized during lactation together with the group of six which had undergone the control operation were remated about 3 weeks after weaning. No difficulty was encountered in mating the thyroidectomized animals, only one of the eight failing to become preg-

nant. One of the six controls also failed to become pregnant. Towards the end of pregnancy several of the thyroidectomized animals showed severe symptoms of tetany, which in one case proved fatal. The remaining cases

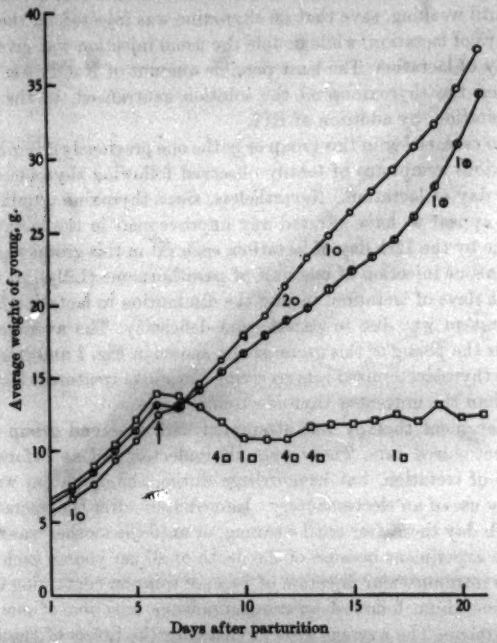

Fig. 2. Average growth curves of pooled litters. □—□ Group of three rats thyroidectomized on the 6th day of lactation and thereafter treated with thyroxine and parathormone (see text). Number of pups at beginning of experiment = 21 (16♂♂, 5♀♀). ⓘ—ⓘ Group of four rats unilaterally thyroidectomized on the 6th day of lactation. Number of pups at beginning of experiment = 32 (14♂♂, 18♀♀). ○—○ Group of six rats which underwent exposure of the thyroids on the 6th day of lactation. Number of pups at beginning of experiment = 44 (18♂♂, 26♀♀). † indicates the last observation before the operation. The numbers underneath the curves refer to the number of pups, belonging to the group indicated by the subscript, which were found dead on the relevant occasion of weighing.

quickly recovered on the injection of one unit of parathormone. Similar observations were made by Nelson & Tobin [1937], who found that removal of the thyroids and the associated parathyroid tissue from

pregnant rats resulted in a high incidence of tetany. It is interesting also to note that Dragstedt *et al.* [1924] found that thyro-parathyroidectomized bitches which were in good condition during the earlier stages of pregnancy often developed severe tetany during the latter stages.

As we have seen, six of the seven thyroidectomized rats which became pregnant survived pregnancy. In all six cases parturition was successfully negotiated, no cases of tetany occurring afterwards. The pups at birth were, however, a little smaller than normal and it was further found that in all cases save one, pregnancy was prolonged above normal by some 24 hr. Only two of these six rats weaned pups, and these at weaning were very much smaller than normal. The young of the other four rats died shortly after birth.

By contrast, all of the five controls which became pregnant lactated normally and three of them weaned litters, the average weights of which fell within the normal range. The other two were thyroidectomized on the 6th day of lactation and used in connexion with another experiment. Up

Table I. Reproduction and lactation after thyroidectomy

No. of rat	Date mated Feb. 1938	Date due to litter March 1938	Date of parturition March 1938	No. of young weaned	Average weight of young at weaning g.	Remarks
(*a*) Rats thyroidectomized on 6th day of previous lactation						
6836	15–16	8–9	10	4♂♂ 3♀♀	20·4	
7056	17–18	11–12	—	—	—	Pregnancy was prolonged and rat died of tetany on 14 March 1938
6806	14–15	8–9	11	0	—	Pups eaten soon after birth
6837	14–15	9–10	10	0	—	All pups dead within 3 days of parturition
6594	17–18	11–12	13	0	—	Pups eaten by mother soon after birth
6599	18–19	12–13	14	1	20·0	
6559	22–23	17–18	19	0	—	All pups dead on day after birth
6439	—	—	—	—	—	Did not become pregnant
(*b*) Rats submitted to control operation on 6th day of previous lactation						
6987	15–16	9–10	9	5♂♂ 3♀♀	43·1	
6396	—	—	—	—	—	Did not become pregnant
6587	16–17	10–11	11	3♂♂ 4♀♀	41·4	
6982	19–20	13–14	14	4♂♂ 3♀♀	37·6	
6350	24–25	18–19	19	—	—	Thyroidectomized on 6th day of this lactation
6807	20–21	14–15	15	—	—	Thyroidectomized on the 6th day of this lactation

to the time of the operation, however, both litters were growing at about the normal rate. Data relating to these two groups are shown in Table I and average growth curves of the litters in Fig. 3.

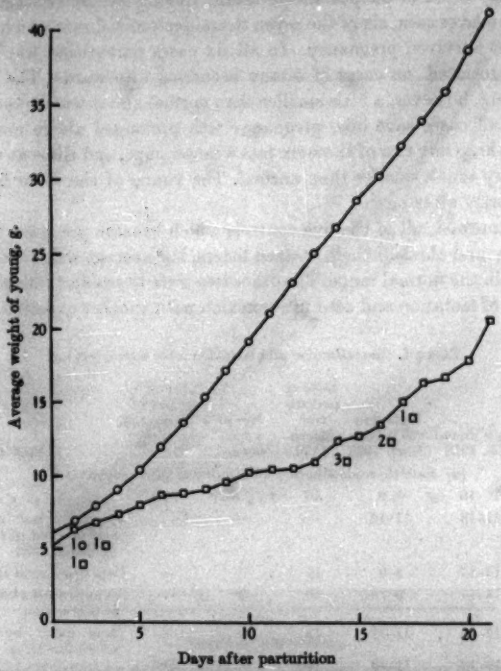

Fig. 3. Average growth curves of pooled litters. □—□ Group of two rats thyroidectomized on the 6th day of the previous lactation. Number of pups at beginning of experiment = 16 (8♂♂, 8♀♀). ○—○ Group of three rats which underwent exposure of the thyroids on the 6th day of the previous lactation. Number of pups at the beginning of experiment = 23 (12♂♂, 11♀♀). The numbers underneath the curves refer to the number of pups, belonging to the group indicated by the subscript, which were found dead on the relevant occasion of weighing.

These results, contrary to those of Nelson & Tobin [1937], clearly show that in previously thyroidectomized animals lactation is very much below normal. It cannot be said that lactation was completely inhibited however, since two out of the six rats weaned pups (rats 6836 and 6599)

while in the other four cases where the young died and were partly eaten by the mothers shortly after birth (rats 6806, 6837, 6594 and 6559) traces of milk could be expressed from the teats.

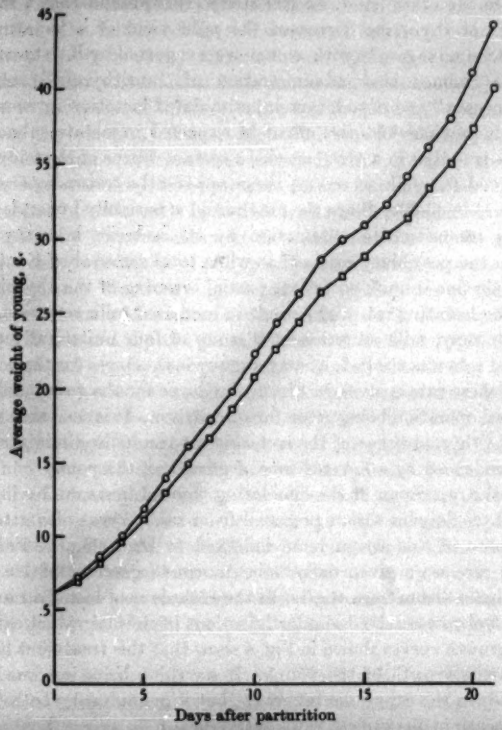

Fig. 4. Average growth curves of pooled litters. □—□ Group of five rats receiving daily injections of anti-thyroglobulin serum from rabbits from the 1st to the 16th day of lactation inclusive. Number of pups at beginning of experiment = 35 (13♂♂, 22♀♀). Two pups died during the experiment. ○—○ Group of five rats receiving daily injections of normal rabbit serum from the 1st to the 16th day of lactation inclusive. Number of pups at beginning of experiment = 31 (10♂♂, 21♀♀). One pup died during the experiment.

The effect of unilateral thyroidectomy during lactation. The preceding results have shown that total removal of the thyroids from the lactating

rat causes a very marked decline in lactation. In line with this observation it is known that administration of moderate doses of thyroxine stimulates lactation in the cow [Graham, 1934*b*; Folley & White, 1936]. On the other hand, de Fremery [1936] found that a relatively high dose of thyroxine decreased the milk yield of a lactating goat. Somewhat similar results with women were reported by Küstner [1934], who also claimed that administration of "antithyroid" substances ("Tyronorman" and di-iodotyrosin) stimulated lactation in women who from their previous histories might be expected to lactate subnormally. If it were true that in a given species a certain degree of thyroid activity was optimal for milk secretion, these apparently contradictory results would be reconcilable. Since the rat thyroid is normally hyperplastic and relatively insensitive to stimulation by the anterior lobe thyrotropic hormone, the possibility exists that while total removal of the thyroids causes a decline in milk secretion, partial lowering of the thyroid secretion in the lactating rat might result in increased milk secretion.

Accordingly, milk secretion in a group of four unilaterally thyroidectomized rats was studied. The average growth curves for the combined litters of these rats is given in Fig. 2, the curve for the group undergoing the control operation being given for comparison. It is seen that removal of half the thyroid tissue of the lactating rat results in diminished lactation as evidenced by a lowered rate of growth of the young.

Partial suppression of the circulating thyroid hormone by injections of anti-thyroglobulin serum prepared from rabbits was also attempted. For supplies of this serum I am indebted to Dr A. S. Parkes. Five lactating rats were given daily subcutaneous injections of 1 c.c. anti-thyroglobulin serum from the 1st to the 16th days of lactation inclusive. Five control rats received similar injections of normal rabbit serum.

The growth curves shown in Fig. 4 show that this treatment had little effect on the growth of the young. If anything lactation was slightly diminished in the experimental group, but it is doubtful whether or not the difference in the growth rates is significant.

Discussion

The results described above show that removal of the thyroids together with the parathyroid tissue associated with them seriously interferes with lactation in the rat. These findings are contrary to those of Nelson & Tobin [1937], but are in line with the results of Graham [1934*a*, *b*], Folley & White [1936], Herman *et al.* [1938] and Folley & Young [1938] and therefore serve to emphasize the importance of the

thyroids in lactation. It is hardly likely that the decline in lactation following thyroidectomy can be ascribed to parathyroid deficiency, since no symptoms of tetany during lactation were observed in thyroidectomized rats, nor did treatment with parathormone effect any improvement in lactation. The failure of thyroidectomized rats to lactate normally when given injections of thyroxine is interesting in view of the fact that thyroxine definitely stimulates lactation in intact animals. This lack of response to thyroxine is unlikely to be due to its inability to act in the absence of thyroid gland tissue, since Gaddum [1929] has shown that synthetic thyroxine will raise the metabolism of the thyroidectomized rat. This aspect of the subject requires further investigation.

The role of the thyroids in lactation has been regarded as relatively unimportant by some workers in this field for the following reason. Lactation cannot be initiated in hypophysectomized guinea pigs possessing the necessary mammary development by injections of refined preparations of anterior lobe lactogenic hormone and simultaneous administration of desiccated thyroid gland [Nelson & Tobin, 1937], nor maintained in guinea-pigs hypophysectomized during lactation by treatment with lactogenic hormone together with thyroxine or anterior lobe thyrotropic hormone [Gomez & Turner, 1937], though in both cases treatment with lactogenic hormone and adrenal cortex hormone is effective. While, however, such experiments serve to emphasize the important role of the adrenal cortex in lactation, they tell us nothing about the role of the thyroids, since there is no evidence that the milk secreted by hypophysectomized animals under the influence of lactogenic hormone and adrenal cortex extract is normal either in amount or composition, nor is it known whether or not treatment with thyroxine in addition to lactogenic hormone and adrenal cortex hormone would have improved lactation still further.

As has been pointed out previously [Folley & Young, 1938], there has been too great a tendency in the past to regard the production by experimental means of a small amount of a fluid with the superficial appearance of milk as normal lactation without regard to the composition of the fluid. In my opinion the best way of obtaining information as to the role played by a given endocrine gland in lactation is by experimenting on animals such as cows and goats, in which it is possible accurately to study the yield and composition of the milk. Studies of lactation in animals such as rats and guinea-pigs will be far more fruitful when suitable techniques for milking out small animals and for microanalysis of milk have been developed.

Summary

1. Removal of the thyroids together with the associated parathyroid tissue from lactating rats causes a marked decline in lactation as judged by the growth rates of sucklings. Rats so thyroidectomized during lactation showed no obvious signs of tetany.

2. No difficulty was experienced in mating thyroidectomized female rats, nor did they fail to become pregnant. Just before parturition many of them exhibited severe symptoms of tetany which disappeared on injection of parathormone. Parturition was normal in all cases but lactation was definitely subnormal.

3. Rats thyroidectomized during lactation and subsequently treated with thyroxine and parathormone lactated no better than untreated thyroidectomized rats.

4. Unilateral thyroidectomy during lactation caused a slight decline in milk secretion.

5. Injection of anti-thyroglobulin serum into lactating rats had very little effect on lactation.

6. The results confirm previous work in emphasizing the importance of the thyroids in lactation.

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J. Physiol. (1938) 93, 413-415 612.799.2:577.176.31

MAXIMUM GROWTH RATE OF CAPON COMB

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(*Received* 11 *June* 1938)

Since McGee [1927] showed that the comb of the capon could be made to develop by the injection of testis extract, much work has been carried out, mostly with a view to the assay of extracts. The difficulty of obtaining large amounts of highly active extracts made impracticable any attempt to investigate the maximum growth rate which can be induced, but now that large amounts of crystalline testosterone are available it has been possible to take up this problem.

Massive prolonged dosage was obtained by the intramuscular implantation of compressed tablets of crystalline testosterone propionate. A group of eight Brown Leghorn capons received implantations of one tablet of 5–16 mg. per bird. The combs were measured at frequent intervals after implantation of the tablets, their size being expressed as the product of length and height. The combs attained their full size, equal to that of normal cocks, in a little over 40 days, and the effect of the tablets lasted for a surprisingly constant period of between 60 and 70 days, after which regression took place. The rate of growth showed no correlation with the weight of the tablet received, from which it was assumed that a maximum growth rate had been stimulated. In order to verify this, another capon (A.C. 21) was given an injection of 100 mg. free testosterone in oil, together with an implantation of a 100 mg. tablet of testosterone propionate. It exhibited a slightly higher growth rate during the first few days only. The figure shows the curves obtained by plotting comb size against time after implantation for these birds, the curve for A.C. 21 being dotted. These curves are of the type of those which may be constructed from the data of Blyth *et al.* [1931], but are steeper and do not fall off at a small comb size. The injections given by these authors were not producing a maximal growth rate. Schoeller & Gehrke [1938] implanted tablets of testosterone and testosterone

propionate up to 2 mg., and also obtained a lower growth rate than we have found with larger tablets. The combs of their birds do not appear to have reached the size of those of normal cocks.

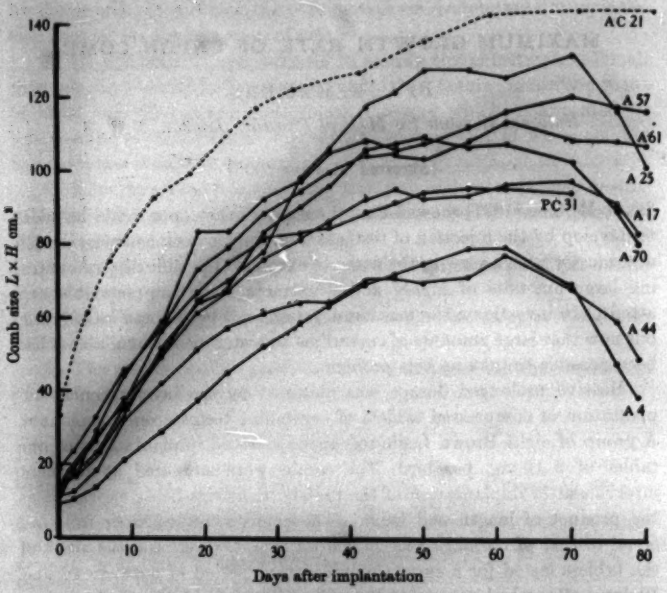

Fig. 1. Growth of capon combs under maximal stimulation, provided by the implantation of tablets of 5–16 mg. testosterone propionate. Dotted line, comb of a bird receiving free hormone (100 mg.) plus a 100 mg. tablet.

From a figure given by Callow & Parkes [1935], of the comb growth of a Brown Leghorn capon, it is seen that they had succeeded in stimulating an equally rapid growth using androsterone. Giving 5 mg. androsterone in oil per day, they found an increase of 85 cm.^{2} ($L \times H$) in 22 days, which is a little greater than that of any of our birds (A.C. 21 grew 75 cm.^{2} in 22 days). Androsterone is therefore capable of producing as rapid a growth as is testosterone.

SUMMARY

Massive prolonged dosage of Brown Leghorn capons has been obtained by the intramuscular implantation of tablets of compressed testosterone propionate. By this method the maximum growth rate of which the comb is capable has been determined, the combs attaining a size equal to that of normal cocks in a little over 40 days.

The testosterone used was kindly provided by Dr K. Miescher and Messrs Ciba.

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J. Physiol. (1938) 93, 416-422 612.62:577.176.31

SOME EFFECTS OF COMPOUNDS OF THE ANDROSTERONE-TESTOSTERONE SERIES ON OVARIECTOMIZED MICE

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(*Received* 22 *June* 1938)

Summaries of the present knowledge relating to the oestrogenic and gynæcogenic activities of members of the androsterone-testosterone series are given by Korenchevsky [1937], for the rat, and by Deanesly & Parkes [1936*a*, 1937] for the rabbit, guinea-pig, rat and fowl. The main substances considered by these authors, and used in the present investigation, are testosterone, androsterone, *trans*-androstenediol (Δ^5-androstene-3-17-*trans*-diol), androstanediol (androstane-3-*cis*-17-*trans*-diol), androstenedione, and *trans*-dehydroandrosterone. None of these has been found to be entirely without gynæcogenic effects. Injection of all but androsterone causes appreciable increase in the weight of the uterus and vagina of the ovariectomized rat [Korenchevsky & Dennison, 1935, 1936*a*, 1937; Korenchevsky *et al.* 1937] and stimulates the vagina of the immature female rat to open precociously [Deanesly & Parkes, 1936*a*]. Testosterone, androstenedione, *trans*-dehydroandrosterone and *trans*-androstenediol stimulate the growth of the immature rabbit uterus [Deanesly & Parkes, 1937], the others listed above not having been investigated. The same four substances are able to feminize the plumage of the Sebright bantam in doses of 1 mg. daily, but androsterone in large doses [Callow & Parkes, 1936], and androstanediol, 1 mg. daily, are without this effect [Deanesly & Parkes, 1937]. Testosterone, androstenediol and *trans*-dehydroandrosterone cause definite mucification of the rat vagina, while androsterone and androstenedione have a slight effect [Korenchevsky & Dennison, 1936*b*; Korenchevsky & Hall, 1937].

Hypertrophy of the female preputial glands in the rat has accompanied the gynæcogenic action of all the androgens [Korenchevsky *et al.* 1935–7]. This and other features indicative of masculinization, such

as enlargement of the clitoris and peri-urethral glands, is an expression of the dual activity of the compounds used. Androgenic activity of this kind is not of necessity inversely proportional to gynæcogenic activity; there is on the contrary a strong tendency for those compounds which are highly androgenic to be the most potent gynæcogens in the series.

Material and methods

The mice used throughout the tests were ovariectomized at 3–6 weeks old and allowed to rest for several months before use. The assays dated 22 September 1937, 16 October 1937 and 16 November 1937 were on mice which had rested for between 2 and 3½ months, that of 2 March 1938 was on mice rested for 6½ months prior to use. Each assay was carried out with groups of four or five mice, injections being given daily for 14 days, at a concentration of 5 mg. of the substance per ml. of nut oil. All injections were therefore of 0·5 mg. in 0·1 ml. of oil, or 1·0 mg. in 0·2 ml. of oil, totalling 7 and 14 mg. respectively.

The mice were killed on the day after the last injection, the uteri, vaginæ and preputial glands dissected out and fixed in aqueous Bouin, and weighed from 70 p.c. alcohol. Vaginal smears were taken on the last two days of the test. Part of the uterine horns was removed at ovariectomy, but no correlation was apparent between the amount removed (always small) and subsequent uterine weight. The body weight of the animals has not been taken into account, as the tests were not intended to be accurately quantitative, and the average body weight of groups in any one test was approximately constant.

Results

Weight of the uterus

The effect of the compounds used on the weight of the uterus is given in Table I, in which they are arranged in descending order of activity. There is a departure from this order in two individual tests, but not to a significant degree, and the order in the table probably represents the true positions of the compounds. It is not suggested, however, that androstenediol is definitely more active than testosterone. In Table II, I have summarized some of the data of Korenchevsky and his co-workers, already quoted, in order to compare the results with those found in rats. Korenchevsky's order of activity is, like my own, in part provisional [Korenchevsky *et al.* 1937, p. 483]. The order of activity found by him differs from mine mainly in the position of testosterone, which he finds to be the most active, and androsterone, found to be the least active by his

TABLE I. Action of members of the androsterone-testosterone series on ovariectomized mice. [Emmens]

Date of assay	Weight of uterus (mg.) 22. ix. 37	16. xi. 37	16. x. 37	2. iii. 38	Weight of preputial glands (mg.) 22. ix. 37	16. xi. 37	16. x. 37	Action on vagina: Smears	Size
Total dose (mg.)	7	7	14	14	7	7	14		
Substance									
trans-Androstenediol	43	77	90	67	10	9	15	Completely cornified, no leucocytes	Hypertrophied
Androstanediol	41	62	54	41	25	23	25	Dioestrus	"
Testosterone	37	48	52	47	15	11	23	Dioestrus with some mucus	"
Androstenedione	20	40	34	27	9	10	19	Dioestrus, but with much mucus	As controls
Androsterone	15	32	30	19	12	9	17	Dioestrus	"
trans-Dehydroandrosterone	16	28	23	14	4	4	7	Some cornification, leucocytes present	Hypertrophied
Controls (no injections)	7	—	8	—	<2	—	<2	Dioestrus	Small, normal castrate

TABLE II. Action of members of the androsterone-testosterone series on ovariectomized rats. [Korenchevsky *et al.*]

Substance	Daily dose mg.	Weight of uterus: Controls	Injected	Weight of preputial glands: Controls	Injected	Weight of vagina: Controls	Injected	Controls average body weight (g.)	Injected average body weight (g.)	Average age days
Androstenediol	0·20	28	51	59	178	127	141	201	187	65
	0·60	28	80	59	145	127	171	201	188	65
Androstanediol	0·49	40	59	83	244	125	180	—	—	105
	0·62	33	99	85	198	81	155	—	—	63
Testosterone propionate	0·167	36	186	77	225	119	249	211	182	61
	0·70	41	240	74	306	142	349	279	237	82
Testosterone	0·167	36	45	77	167	119	139	211	155	56
	0·70	36	113	77	145	119	194	211	204	56
	0·167	41	58	74	140	142	184	279	277	106
	0·70	41	138	74	165	142	221	279	280	106
Androstenedione	0·50	30	54	75	161	132	181	222	233	68
	1·00	30	53	75	189	132	196	222	221	68
	3·00	30	77	75	260	132	213	222	224	68
	0·50	35	50	67	157	138	209	260	280	79
	3·00	35	101	67	204	138	235	260	256	79
Androsterone	0·90	33	32	—	—	115	130	—	—	118
	1·80	33	39	—	—	125	124	—	—	131
	3·60	34	47	65	274	94	124	—	—	63
trans-Dehydroandrosterone	1·00	33	56	76	190	130	143	218	213	
	2·00	33	57	76	174	130	152	218	208	61–77
	4·00	33	69	76	146	130	163	218	210	

tests. These differences are almost certainly dependent on the use of different test animals. *trans*-Androstenediol, *trans*-dehydroandrosterone, androstanediol and testosterone were found by Deanesly & Parkes [1937] to stimulate development of the immature rabbit uterus with that order of activity, indicating another interspecific difference in sensitivity.

A feature of the assays of the activity of these compounds is the large inter-assay variation in response at different dates. The uterus weight resulting from the injection of a total of 14 mg. of each compound for the period beginning 2 March 1938 was actually, in every instance, less than that which resulted from the injection of 7 mg. total over the period dated 16 November 1937. In this particular case, the large interval between ovariectomy and use (6½ months) of the mice on the higher dosage might be thought to be the cause of the discrepancy. However, mice rested for approximately equal periods and receiving the same total dosage (assays dated 22 September 1937 and 16 November 1937) gave widely different responses, whilst preserving the same order of activity within each test. In view of the shift in sensitivity to œstrone which has been demonstrated for mice of the same stock kept under the same conditions as these in question [Emmens, 1938], it seems likely that a similar time-to-time variation occurs in these assays.

Effect on vagina

The vaginæ were found to be hypertrophied, in comparison with the controls, in all cases but those of mice receiving androsterone and androstenedione (Table I). From Table II it will be seen that Korenchevsky obtained an increase in vaginal weight with the highest dose (3·6 mg. daily) of androsterone, and with all doses of androstenedione. All of the compounds were active on rats.

Complete cornification occurred in the vaginal smears of all mice receiving *trans*-androstenediol, as Deanesly & Parkes [1937] found. Korenchevsky (private communication) has not been able to produce complete cornification in rats with this compound, using doses up to 0·6 mg. daily for 15 days. In my experiments on mice some cornification was found in the smears of those receiving *trans*-dehydroandrosterone, but not regularly. Androstenedione caused mucification to a marked degree, testosterone a slight mucification. This agrees with Deanesly & Parkes' [1937] findings for their action on the vagina of the immature rat. Androsterone and androstanediol produced no change from the normal dioestrous smear. Korenchevsky & Hall [1937] record only slight mucification in rats injected with androstenedione.

Preputial gland weight

Like the uterus, the female preputial glands of the ovariectomized mouse are capable of undergoing great hypertrophy. The preputials of uninjected animals regularly weigh less than 2 mg.; those injected with excess of androstanediol attain an average of 25 mg. The order of activity on these glands is shown in Table I. Androstanediol is outstandingly active in comparison with the other compounds. Korenchevsky *et al.* [1937] found androstenedione to be about as active as androstanediol on rats, these being together the most active of those tested.

Regarding hypertrophy of the female preputials as a response to androgenic activity, it is of interest that the activities of these compounds in producing growth of the capon comb, and the seminal vesicles and prostate of the castrated male rat, do not correlate well with their activities on the preputial gland. Table III gives them in descending order

TABLE III. Members of the androsterone-testosterone series arranged in descending order of activity on the preputials of rats and mice, the capon comb and castrated male rat. Data for rats from Korenchevsky *et al.* [1935–7], for capons from Deanesly & Parkes [1936*b*]

Rats Female preputial glands	Mice Female preputial glands	Capons γ per I.U. approx.	Castrate male rats, equivalent amounts in γ
Androstanediol Androstenedione	Androstanediol Testosterone	Testosterone 17 Androstanediol 33	Testosterone 8 Androstanediol 21
trans-Androstenediol Testosterone Androsterone?	*trans*-Androstenediol Androstenedione Androsterone	Androstenedione Androsterone *trans*-Androstenediol 235*	100 Androsterone 170
trans-Dehydro-androsterone	*trans*-Dehydro-androsterone	*trans*-Dehydro-androsterone 300	*trans*-Dehydro-androsterone 940

* Koch [1937] quotes as an alternative to Deanesly & Parkes' value for *trans*-androstenediol those of Butenandt & Tscherning, and Tschopp & Ruzicka, which are 650 and 525 γ per I.U. respectively.

of activity on the preputial glands of rats (from Korenchevsky *et al.* 1935–7], and of mice, on the combs of capons [Deanesly & Parkes, 1936*b*] and on castrate male rats [Korenchevsky & Dennison, 1936*a*].

Multiple activity

Table IV summarizes the data available concerning the relative degree of activity of the compounds under discussion. The number of plus signs indicates relative activity only, and is a rough guide to the potency of any particular compound. The sources of data used in constructing Table IV have already received mention.

TABLE IV. Comparative activity of compounds on different test animals. Activity indicated by plus signs, but not quantitatively. The sign +_ indicates very slight activity.

Substance	Spayed mouse uterus wt. [Emmens]	Spayed rat uterus wt. [Korenchevsky, etc.]	Immature rabbit uterus wt. [Deanesly & Parkes]	Immature rat vagina opening [Deanesly & Parkes]
trans-Androstenediol	+ + + +	+ + +	+ + + +	+ + + +
Androstanediol	+ + +	+ + +	—	+ + +
Testosterone	+ + +	+ + + +	+	+
Androstenedione	+ +	+ +	+ +	+ +
Androsterone	+	+_	—	+_?
trans-Dehydroandrosterone	+_	+	+ + +	+ + +

Substance	Spayed mouse preputial wt. [Emmens]	Spayed rat preputial wt. [Korenchevsky, etc.]	Capon comb growth [Deanesly & Parkes]	Castrate male rat [Korenchevsky, etc.]
trans-Androstenediol	+ +	+ + +	+	—
Androstanediol	+ + + +	+ + + +	+ + +	+ + +
Testosterone	+ + +	+ +	+ + + +	+ + + +
Androstenedione	+ +	+ + + +	+ +	—
Androsterone	+ +	+	+ +	+ +
trans-Dehydroandrosterone	+	+	+	+

The relative activities on ovariectomized rats and mice do not disagree markedly, but the tests of Deanesly & Parkes on the immature rabbit uterus and the immature rat vagina have shown a different order for these animals. It is remarkable that in both of their tests testosterone takes a low place, and *trans*-dehydroandrosterone a higher one, the reverse of the findings with ovariectomized rats and mice.

There is a somewhat greater discrepancy between the activity revealed by female preputial gland weight in ovariectomized rats and mice. Neither order of activity corresponds with that found by the usual capon tests, or with Korenchevsky's rat tests for androgenic activity, which agree with each other excellently as far as order of activity is concerned. It is evident, therefore, that female preputial gland hypertrophy does not indicate quite the same kind of activity as do these tests. It correlates rather better, but not well, with activity in increasing uterus weight.

SUMMARY

1. The comparative effects of androgens on the uterus, preputial glands and vagina of the ovariectomized mouse are reported.

2. *trans*-Androstenediol, androstanediol, testosterone, androstenedione, androsterone and *trans*-dehydroandrosterone cause increase in the weight of the uterus in that descending order of activity.

3. *trans*-Androstenediol, androstanediol, testosterone and *trans*-dehydroandrosterone cause increase in vaginal size, only the first-named causing complete cornification. Androstenedione will cause mucification; testosterone slight mucification.

4. The order of activity in causing hypertrophy of the female preputial glands is: androstanediol, testosterone, *trans*-androstenediol, androstenedione, androsterone (the last three of approximately the same activity), and *trans*-dehydroandrosterone. This is not the order of activity of the same substances in the capon comb growth or castrate male rat tests.

My best thanks are due to Dr K. Miescher and Messrs Ciba for their generosity in providing the compounds used in the present investigation, and to Dr A. S. Parkes for his constant help during the work.

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J. Physiol. (1938) 93, 423-429 612.824.1:547.781.5

THE EFFECT OF THE SUBARACHNOID ADMINISTRATION OF HISTAMINE ON THE RATE OF ABSORPTION OF ISOTONIC SALINE SOLUTION IN THE DOG

By T. H. B. BEDFORD

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(*Received* 6 *July* 1938)

A study of the effect of the subarachnoid administration of histamine was first made by Forbes *et al.* [1929]. These workers observed the cerebral vessels through a glass window fixed in the cranium of cats under amytal anæsthesia. Ringer's solution containing histamine was perfused under the glass window. The pial vessels bathed in the solution were observed to dilate sharply. No significant change was observed in the systemic blood pressure or in the pressure of the cerebrospinal fluid. They found in further experiments that ether anæsthesia was unsuitable for studying the effect of histamine on the cerebral vessels; the vessels were dilated by the ether.

Cushing [1932] injected histamine into the lateral ventricles. He found that a dose corresponding to five times that needed to produce flushing effects when given intravenously had no appreciable effect when injected into the ventricle of a monkey. In the human subject, a dose twice as large as that which evokes an almost immediate and prolonged flush when injected into the muscles was found to cause facial pallor rather than flushing when injected into the ventricles. The pallor was accompanied by headache, some nausea and possibly by a temporary increase in gastric acidity. It is evident from these experiments that the effects which follow the intraventricular administration of histamine differ profoundly from those observed after intravenous injection.

An attempt has been made to determine whether the introduction of histamine into the subarachnoid space has any influence on the rate of absorption of Tyrode solution.

Experimental procedure

The animals used in these experiments were dogs varying from 7 to 10 kg. in weight and from 2 to 4 years in age. In one group of experiments the animals were anæsthetized with intratracheal ether. In another group amytal (iso-amyl-ethyl barbituric acid) was the anæsthetic. The amytal was given in a 5 p.c. solution in water. A quantity equivalent to 0·08 g. per kg. of body weight was found to be the optimum dose. After intraperitoneal injection, the animals were kept quiet in a darkened room for a standard period of 20 min. They were then found to be in a satisfactory state of anæsthesia. In a number of experiments the animals were lightly anæsthetized with ether, and amytal in a 1 p.c. solution in water was introduced through a tube into the stomach. A proportion of 0·12 g. amytal per kg. of body weight was found to be the optimum when the drug was administered by the stomach. The animals speedily recovered from the ether anæsthesia; the amytal anæsthesia was usually established after an hour.

The isotonic solution used throughout the experiments was Tyrode solution. Its rate of absorption was determined by means of an apparatus originally devised by Mortensen & Weed [1934] for determining the rate of absorption of solutions from the subarachnoid space. It is unnecessary to give an account of this apparatus for it has already been described in detail [Bedford, 1938]. The Tyrode solution was introduced through a needle in the cisterna magna and its rate of absorption at a constant pressure of 300 mm. saline solution determined. A pressure of 300 mm. was chosen because control experiments had demonstrated that a reasonable volume of fluid was absorbed and there was no evidence of interference with normal functions at this pressure level. Pressure readings were taken at intervals of 3 min. and the initial control period was never less than 15 min. After the normal rate of absorption had been determined, a closed reservoir system containing the histamine in Tyrode solution was interposed between the needle in the cisterna magna and the recording apparatus. The loss of fluid from the subarachnoid space was avoided as far as possible during this procedure. Tyrode solution containing histamine was then introduced at a constant pressure of 300 mm. and its rate of absorption observed. The preparation of histamine used in these experiments was histamine acid phosphate (Burroughs, Wellcome). It was introduced in concentrations of 1 : 100,000, 1 : 50,000 and 1 : 25,000. The average duration of an experiment was 1½ hr. At the end of an experiment a suspension of Indian ink (Reeves) was introduced at a

pressure of 300 mm. saline through a needle in the cisterna magna for about 15 min. A dissection was then made to discover whether there had been any leakage in the region where the needle passes through the dura and the arachnoid. Experiments in which leakage had occurred were discarded.

The blood pressure in the femoral artery was recorded throughout the experiments.

The effect of the subarachnoid administration of histamine on the rate of absorption of Tyrode solution in animals under ether anæsthesia

Ether anæsthesia prolonged for 2 hr. had no appreciable influence on the rate of absorption of Tyrode solution. The addition of histamine to

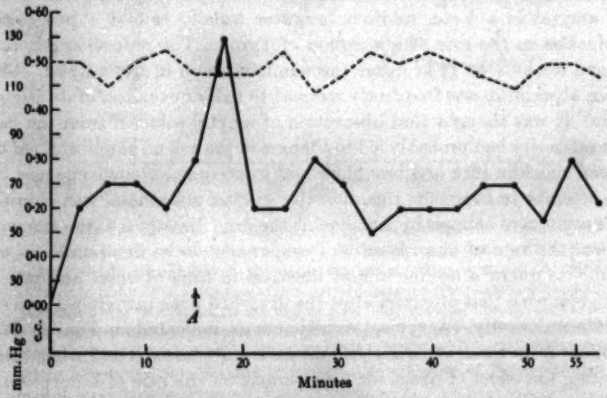

Fig. 1. The effect of histamine on the rate of absorption of Tyrode solution from the subarachnoid space. The reservoir containing histamine in Tyrode solution in a concentration of 1 : 25,000 was connected with the needle in the subarachnoid space at *A*. During this procedure a quantity of fluid escaped from the subarachnoid space. This is the explanation of the excessive inflow during the first 3 min. after reconnecting. The rate of inflow was determined at a constant pressure of 300 mm. saline solution throughout the experiment. It is expressed in terms of the volume of solution entering the subarachnoid space during periods of three minutes. The systolic pressure in the femoral artery is recorded in millimetres of mercury. The anæsthetic is ether.
•—• Volume of Tyrode solution absorbed. ---- Systolic pressure in femoral artery.

the solution in concentrations of 1 : 100,000, 1 : 50,000 and 1 : 25,000 was without significant effect when introduced for periods varying from $\frac{1}{2}$ to

1 hr. The average rate of inflow of the histamine containing solution in the ten experiments was 0·061 c.c. per min. The systemic blood pressure remained unchanged. A typical experiment is indicated in Fig. 1. In a number of experiments cerebrospinal fluid was withdrawn and replaced by Tyrode containing histamine. Even the replacement of 1 c.c. of cerebrospinal fluid by a similar quantity of Tyrode containing histamine in a concentration of 1 : 25,000 was found to be without effect on the rate of absorption from the subarachnoid space. The systemic blood pressure was also uninfluenced.

The effect of the subarachnoid administration of histamine on the rate of absorption of Tyrode solution in animals under amytal anæsthesia

Control experiments showed that the intraperitoneal administration of amytal in a 5 p.c. solution in water usually caused a progressive reduction in the rate of absorption of Tyrode. The reduction generally began about 1 to 1½ hr. after the administration of the amytal. After 2 hr. absorption was frequently reduced to half or even less of its original rate. It was thought that absorption of amytal solution from the peritoneal cavity had probably led to changes in the colloid equilibrium of the blood which in turn had brought about intracranial volume changes. In an attempt to overcome this difficulty amytal anæsthesia was obtained in a number of animals by introducing the drug directly into the stomach. When the rate of absorption of Tyrode came to be determined it was found to follow a decline almost identical in time of onset and rate of progress with that observed after the drug had been introduced into the peritoneal cavity. A typical experiment is indicated in Fig. 2. It is evident from these experiments that care must be exercised when interpreting the effect of drugs for long periods on the rate of absorption of saline solution in animals under amytal anæsthesia.

According to Forbes *et al.* [1929] amytal, unlike ether, does not tend to mask the action of histamine on the blood vessels of the brain. An attempt was accordingly made to study the effect of histamine on the rate of absorption when introduced into the subarachnoid space of animals under amytal anæsthesia. The interpretation of the results of these experiments was rendered somewhat difficult owing to the fact that amytal itself even when administered by the stomach causes a progressive reduction in the rate of absorption. As far as could be determined from a careful comparison with control experiments, histamine when introduced into the subarachnoid space of animals under amytal anæs-

thesia has no significant effect on absorption. Fifteen experiments were performed in all. The systemic blood pressure was also uninfluenced by the histamine in these experiments.

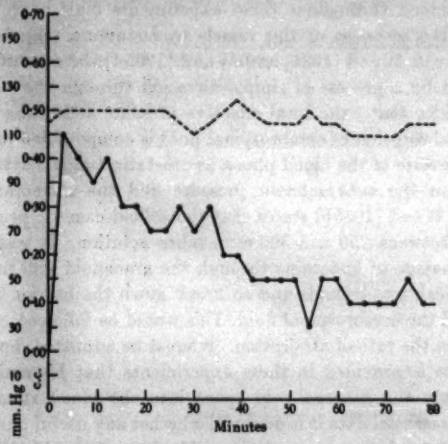

Fig. 2. The effect of amytal on the absorption of Tyrode from the subarachnoid space. The animal was lightly anæsthetized with ether and amytal in a proportion of 0·12 g. per kg. of body weight introduced into the stomach. The amytal was administered in a 1 p.c. solution in water. The animal speedily recovered from the ether anæsthesia and amytal anæsthesia was established at the end of ½ hr. Recording of absorption was begun 1 hr. after the administration of the amytal. The rate of inflow was determined in the same way as in the experiment indicated in Fig. 1. The systolic pressure in the femoral artery is recorded in millimetres of mercury. •—• Volume of Tyrode solution absorbed. - - - - Systolic pressure in femoral artery.

Discussion

The results obtained in the above experiments were contrary to those anticipated. It was considered on theoretical grounds that the introduction of histamine into the subarachnoid space would probably lead to a diminution in the rate of absorption. It seemed possible for this to be effected in two ways. (1) Forbes *et al.* [1929] have shown that the direct application of histamine to the cerebral blood vessels leads to an immediate dilatation even in animals under ether anæsthesia. Histamine when introduced into the subarachnoid space is probably restricted in its

action to the superficial vessels of the brain. It was considered however that the dilated blood vessels would obstruct to some extent the circulation of the cerebrospinal fluid and so retard absorption. It must be remembered that the relatively high pressure of 300 mm. saline which was maintained throughout these experiments may have limited the extent of the response of the vessels to histamine. (2) According to Mortensen & Weed [1934] and Weed [1935*a*] the cerebrospinal fluid is absorbed by a process of simple filtration through the cerebral villi. They consider that "the total effective pressure activating the normal process of absorption of cerebrospinal fluid is compounded of the colloid osmotic pressure of the blood plus a hydrostatic factor derived from the difference in the subarachnoid pressure and the intracranial venous pressure". Weed [1935*b*] states that the colloid osmotic pressure of the blood lies between 250 and 300 mm. saline solution. It was considered that the passage of histamine through the arachnoid villi might render them permeable to colloids and so break down the barrier between the plasma and the cerebrospinal fluid. This would be followed by a marked reduction in the rate of absorption. It must be admitted, however, that no evidence is provided in these experiments that histamine actually escaped from the subarachnoid space into the blood stream. In the absence of essential data it is doubtful whether any useful purpose will be served by speculating as to the possible fate of the histamine. These experiments also provide no explanation for the action of amytal, when administered by the stomach, on the rate of absorption of Tyrode. They emphasise however the need for an anæsthetic which is without influence on absorption and which does not tend to mask the response of the cerebral vessels to histamine.

Any explanation of the phenomenon of meningismus on the basis of an escape of histamine into the subarachnoid space is rendered improbable by these experiments.

Summary

A study has been made of the effect of the subarachnoid administration of histamine on the rate of absorption of Tyrode solution from the subarachnoid space of dogs under ether and under amytal anæsthesia. In neither instance had the histamine any influence on the rate of absorption. The systemic blood pressure was also uninfluenced. While control experiments showed ether to be without effect, amytal even when administered by the stomach caused a progressive reduction in the rate of absorption.

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J. Physiol. (1938) 93, 430-437

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THE EFFECT OF HYPERTONIC SOLUTIONS ON GASTRIC SECRETION AND INTRA-OCULAR PRESSURE

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(*Received* 8 *July* 1938)

The mechanism of formation of the aqueous humour has been studied by many workers. Hertel [1914] showed that the injection of hypertonic solutions of such substances as sodium chloride, sugar and urea, was followed by a fall in the intraocular pressure. These observations have been confirmed by Sansum [1917], Duke-Elder [1926], Fremont-Smith & Forbes [1927]. A similarity in the behaviour of the intraocular and cranial pressure following the intravenous injection of hypertonic solutions has been noted. A lowering of intracranial pressure has been described by Weed & McKibben [1919, 1922], Haden [1919], Cohen [1924]. These changes in the fluid content of the eye and the brain were explained by assuming dehydration following an increase in the osmotic pressure of the blood as a result of the hypertonic intravenous injection. As a result of these experiments the aqueous humour and cerebro-spinal fluid were considered to be dialysates of blood plasma. Robertson [1938] has criticized the suggestion that the aqueous humour is a dialysate. He has shown that during the intravenous injection of 30 p.c. NaCl a great increase in blood volume takes place, but as soon as the injection is completed the vascular system at once begins to rid itself of the fluid attracted from the tissues. In half to three-quarters of an hour the blood volume is back to normal. In a series of experiments it was found that after an intravenous injection of 30 p.c. NaCl there is a gradual and well-marked fall in the intraocular pressure, reaching its zero in about an hour, remaining at this level for a few hours. At this time, however, blood volume has returned to normal. The fluid exchange in the eye must, therefore, differ from that of the tissues. This

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failure of the intraocular pressure to follow the general dehydration and fluid equilibrium throughout the body suggested that some mechanism other than that of dialysis must be present for the formation of the aqueous humour. In the following experiments changes in intraocular pressure have been compared with alterations in the gastric secretion.

Methods

Adult male cats, starved for 24 hr., were maintained under "nembutal" (sodium ethylbutylbarbiturate) anæsthesia throughout all the experimental procedures. Respiration was kept at a steady rate by means of a respiratory pump and tracheal intubation. Blood pressure was recorded from the left carotid artery, and arterial blood for the estimation of hæmoglobin was removed from one femoral artery. A needle was inserted into the anterior chamber of the right eye and the intraocular pressure recorded by a mercury monometer. Hæmoglobin was determined by methods previously described [Robertson, 1935]. A gastric fistula was established after ligating the pylorus, and a technique similar to that formerly reported [Cutting *et al.* 1937] was followed. Gastric stimulation was maintained by the subcutaneous injection of 1·0 mg. histamine at hourly intervals. The hypertonic solutions were injected into either the external jugular or the femoral veins. Control experiments on uninjected cats showed that the intraocular pressure remained constant under these experimental conditions. In three preliminary experiments the effect of 30 p.c. NaCl and 50 p.c. glucose was observed on the gastric secretion and intraocular tension of the same animal. In the remaining experiments these effects were observed on different animals.

Effect of 30 *p.c.* NaCl 5 *c.c. per kg. and* 2 *c.c. per min.* In animals where the gastric secretion and intraocular pressure have been measured simultaneously or when measured separately on different animals, it has been found that following 30 p.c. NaCl there is a striking fall in the pressure in the eye and in the volume and acidity of gastric juice. These changes have been repeatedly produced and a typical composite chart is shown in Fig. 1. It will be seen that the hæmoglobin became markedly diluted at the end of the injection, but then rapid concentration occurred with a return to normal in 30–45 min. Intraocular pressure, on the other hand, showed a definite gradual fall quite independent of blood pressure, which reached its lowest level in about 60 min., and remained low for at least the next hour. The volume and acidity of the gastric juice were always lowered, and frequently cessation of secretion occurred. The duration of

this inhibition varied but may extend over 3–4 hr. In Fig. 1 the total 30 min. secretion of acid is expressed as a factor—the product of total acid and volume over 100. When it is considered that the stomach is

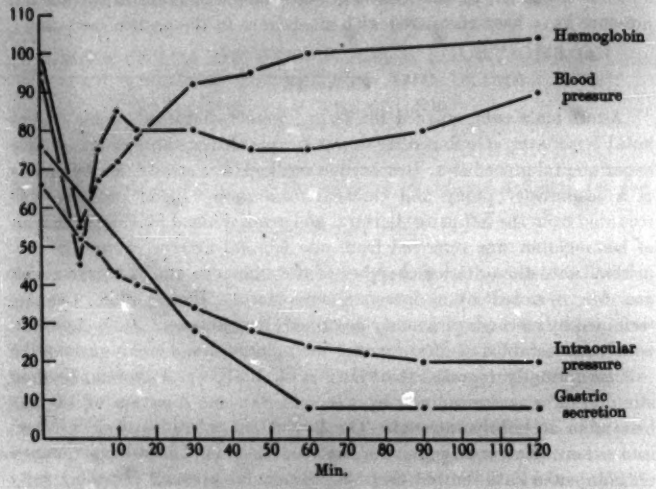

Fig. 1. Hæmoglobin, intraocular pressure, blood pressure and total gastric secretion following 30 p.c. NaCl—5 c.c. per kg.

TABLE I. Gastric secretion following 30 p.c. NaCl—5 c.c. per kg.

	C2			C72		
Time min.	Volume c.c.	Free HCl c.c. *N*/10 per 100 c.c.	Total acid c.c. *N*/10 per 100 c.c.	Volume c.c.	Free HCl c.c. *N*/10 per 100 c.c.	Total acid c.c. *N*/10 per 100 c.c.
0	1 mg. histamine subcutaneously and at every hour subsequently					
30	2·5	132	140	1·6	42	60
60	3·5	138	145	3·1	128	138
90	4·2	136	142	2·3	145	151
90	30 p.c. NaCl intravenously—5 c.c. per kg.—2 c.c. per min.					
120	1·4	142	148	1·0	128	140
150	1·9	88	102	0·43	100	116
180	3·8	54	64	0·24	61	81
210	5·8	76	87	0·28		
240	3·6	102	112	0·21	51	68
270	3·6	111	122	0·39		

receiving a continuous stimulation from histamine, the most powerful gastric stimulus known, the inhibiting action of hypertonic NaCl must be great. In Table I the inhibiting effect on gastric acidity and volume of

juice may be seen. C2 showed an unusually slight and transitory lowering of volume and acid content, but in C72 the effect was more marked and prolonged.

Effect of 50 p.c. glucose, 17 c.c. per kg. and 7 c.c. per min. The effect of 50 p.c. glucose in a dose of 17 c.c. per kg. given at the rate of 7 c.c. per min. has been observed in a series of experiments comparable with those using 30 p.c. NaCl. The total osmotic pressure of the volume of glucose

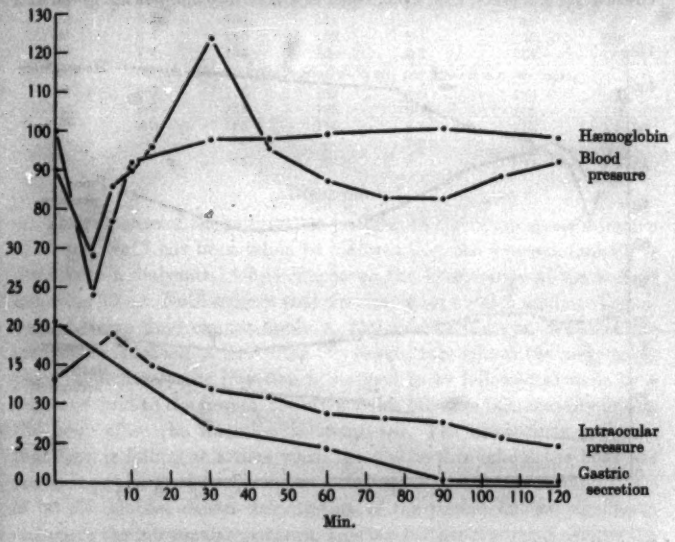

Fig. 2. Hæmoglobin, intraocular pressure, blood pressure and total gastric secretion following 50 p.c. glucose—17 c.c. per kg.

solution was roughly equivalent to that of the volume of 30 p.c. NaCl in the previous experiment. Some difficulty was experienced in keeping the animal alive following such massive injections of glucose, especially in the gastric experiments where histamine had to be given. A typical chart is shown in Fig. 2. The hæmoglobin became greatly diluted, rapidly returned to normal as it did after the injection of 30 p.c. NaCl. The effect on the intraocular pressure differed slightly from that produced by 30 p.c. NaCl, in that there was a definite rise in pressure during the course of the injection—possibly related to an alteration in venous pressure.

Following this, however, there was a gradual fall to a low level similar to, but more delayed than that following 30 p.c. NaCl. Gastric secretion was inhibited. In this case the acidity fell abruptly after the glucose injection, but the volume showed a partial reduction for the next hour and thereafter was inhibited. The volume apparently behaved in a closely parallel fashion to the intraocular pressure.

Effect of 50 p.c. glucose or 9 p.c. NaCl—5 c.c. per kg. at 2 c.c. per min. The effect of 50 p.c. glucose or 9 p.c. NaCl in a dose of 5 c.c. per kg. given at a

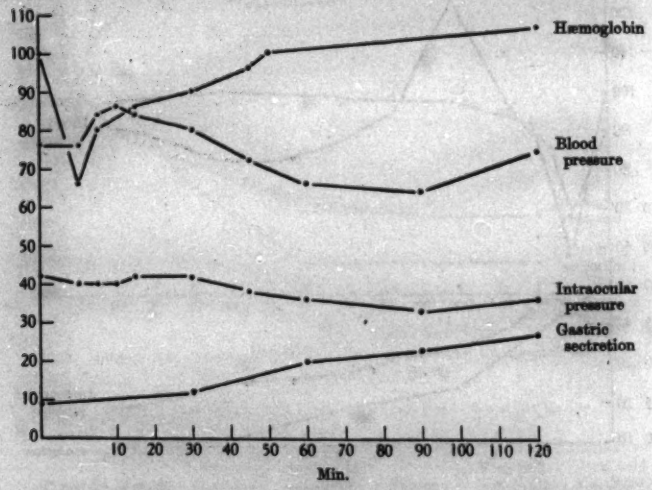

Fig. 3. Hæmoglobin, intraocular tension, blood pressure and total gastric secretion following 9 p.c. NaCl—5 c.c. per kg. and 50 p.c. glucose—5 c.c. per kg. The action of 9 p.c. NaCl (5 c.c. per kg.) was similar.

rate of 2 c.c. per min. has been examined, as these two solutions have approximately the same osmotic pressure. The action of these two solutions has been found to be similar in all respects and a typical graph may be seen in Fig. 3. There was a dilution of hæmoglobin which was not so marked as in the preceding experiments due to the relatively low osmotic pressure of the solutions. A rapid return to normal was again apparent. With these concentrations there was usually no effect on either the intraocular pressure or gastric secretion. The effect on the gastric acidity

and volume of juice in two cats is shown in Table II. In these cases, the 50 p.c. glucose did not alter the secretion and the response is identical with that found in control experiments using histamine alone.

TABLE II. Gastric secretion following 50 p.c. glucose—5 c.c. per kg.

	C19			C71		
Time min.	Volume c.c.	Free HCl c.c. *N*/10 per 100 c.c.	Total acid c.c. *N*/10 per 100 c.c.	Volume c.c.	Free HCl c.c. *N*/10 per 100 c.c.	Total acid c.c. *N*/10 per 100 c.c.
0	1 mg. histamine subcutaneously and at every hour subsequently					
30	2·7	103	114	1·9	45	63
60	6·3	146	152	3·1	124	136
90	7·9	154	160	3·3	144	152
90	50 p.c. glucose intravenously—5 c.c. per kg.—2 c.c. per min.					
120	8·0	156	162	4·0	160	166
150	8·9	163	169	6·2	165	172
180	9·5	163	170	6·8	170	176
210	—	—	—	7·9	168	174
240	—	—	—	9·7	166	172

DISCUSSION

The response of the intraocular pressure to the intravenous injection of 30 p.c. NaCl has been taken to indicate that the aqueous humour is essentially a dialysate. Observations on the interchange of tissue fluid following 30 p.c. NaCl suggest that the simple laws which apply to formation of tissue fluid cannot apply to the fluid in the eye. Whereas the greatest withdrawal of fluid from the tissues throughout the body takes place immediately the injection is stopped to be followed at once by a return of fluid to the tissues, the intraocular pressure falls steadily during the hour after the injection is completed. The intraocular pressure, therefore, is falling at a time when the tissues throughout the body are increasing their fluid content. The intravenous injection of suitable doses of 50 p.c. glucose causes dehydration of the tissues but no significant change in the intraocular pressure. This is a further argument against the dialysate nature of the aqueous humour. That the general reaction is an osmotic one is shown by the similar behaviour of the intraocular pressure to 50 p.c. glucose given in large enough doses to equal 30 p.c. NaCl. For many years there has been a controversy regarding the nature of the aqueous humour which has in turn been considered a secretion, dialysate, transudate, and exudate. Observations on the interchange of tissue fluid suggest that the aqueous humour can no longer be regarded as a dialysate. It must be pointed out, however, that the measurement of the intraocular pressure does not necessarily indicate what type of change is occurring in the intraocular fluid. Assuming the blood pressure is unaltered, the intraocular pressure is a mean of the volume of aqueous

humour produced and the volume eliminated. A fall in the intraocular pressure may therefore be due to a diminished rate of production, an increased rate of elimination over production, or a combination of both these factors. During the injection of 30 p.c. NaCl it is probable that any fall which occurs in the intraocular pressure is in great measure due to an increased rate of elimination of the aqueous humour, for under similar conditions Weed found that the cerebro-spinal fluid was absorbed by two routes in addition to the normal mechanism through the arachnoid villi. Immediately after the completion of the injection, however, osmotic equilibrium is established in the dialysates throughout the body, so that any fall in the intraocular pressure which occurs after the injection cannot be explained on the purely physical laws governing osmosis. It would not seem improbable, therefore, that the continued fall in the intraocular pressure after the completion of the injection is due to a failure in the production of the aqueous humour. There seems no reason to believe that the rate of elimination of the aqueous humour over production should be increased when the force (hypertonic NaCl) which caused the transfer of fluid had ceased to act.

In the case of the stomach a study was made of the secretion of HCl and the volume of gastric juice produced, and it was noted that the behaviour of one intimately followed that of the other, although it is conceivable this may not always be so under different experimental conditions. In comparing the behaviour of the stomach with that of the eye it would seem less open to criticism to compare the curve of the intraocular pressure with that of the volume of gastric juice and the curve of the concentration of hydrochloric acid with that of the secretion of the eye. There is, as yet, little evidence as to the nature of a specific secretion in the eye, although recently Meyer & Palmer [1936] have described the presence in the eye of a specific secretion—a polysaccharide called polyuronic acid.

In the above experiments the striking similarity in the behaviour of the intraocular pressure with that of the gastric secretion has been noted. Since the formation of gastric juice is a typical secretion, it suggests that some sort of secretory activity in the eye may be concerned with the control of the intraocular pressure.

Summary

30 p.c. NaCl and 50 p.c. glucose solutions of equal osmotic pressure both cause a dilution in hæmoglobin which rapidly returns to normal. Intraocular pressure is markedly lowered and remains so for some hours.

The gastric acidity and volume of juice evoked by histamine are inhibited and may remain so for some time.

9 p.c. NaCl and 50 p.c. glucose solutions of equal osmotic pressure both cause a similar transitory dilution in hæmoglobin. They have little or no effect on intraocular pressure or gastric secretion.

The findings indicate that dialysis is not a satisfactory explanation of the formation of the aqueous humour. A similarity in response to hypertonic solutions between gastric secretion and intraocular pressure suggests that a secretory process in the eye may play some part in controlling intraocular pressure.

It is a pleasure to thank Prof. E. C. Dodds for his criticism and interest.

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PROCEEDINGS

OF THE

PHYSIOLOGICAL SOCIETY

14 *May* 1938

Acetylcholine and synaptic transmission in a ganglion. By F. C. MacIntosh.[1] (Introduced by H. H. Dale.) (*From the National Institute for Research, Hampstead*)

In a recent paper Lorente de Nó [1938] describes his repetition of the experiments of Feldberg & Gaddum [1934] and Feldberg & Vartiainen [1934], on the output of acetylcholine from the perfused superior cervical ganglion of the cat. In some experiments he obtained results similar to theirs, but in others the output of acetylcholine was irregular and bore no clear relation to stimulation of the preganglionic fibres. It sometimes appeared in the venous fluid during periods with no stimulation, or during stimulation of the vagus trunk or the sympathetic postganglionic branches.

I find that a good rate of perfusion of this ganglion, 0·5–1 c.c. per min., can be obtained and continued for some hours without perceptible œdema, by using a small pump to produce a pulsatile pressure and a 10 p.c. dilution of heparinized cat's blood in Locke's solution instead of plain Locke's solution, eserine being added in the proportion of $1 : 2{\cdot}5 \times 10^5$. This method was demonstrated at the Physiological Society meeting at Hampstead on 12 February 1938. Under such conditions I have regularly observed a clear correspondence between the output of acetylcholine and the periods of stimulation of the preganglionic nerve, at all stages of the perfusion. The output begins with such stimulation, continues during its progress, and ceases quickly after it; while the venous fluid remains free from detectable acetylcholine in the absence of stimulation, and during stimulation of either the vagus trunk or the sympathetic postganglionic branches. The earlier findings are thus fully confirmed, and their significance, which Lorente de Nó has called into question, is reinforced.

[1] With a Travelling Fellowship from the Royal Society of Canada.

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Transmission of nerve impulses in the central nervous system.

By D. NACHMANSOHN[1]. (*From the Laboratoire de Physiologie générale de la Sorbonne, Paris*)

For assuming a transmitter function of acetylcholine at the synapses of the central nervous system it is necessary that the acetylcholine liberated should be removed during the refractory period of nerve cells. This condition is fulfilled at the neuromuscular junction [Marnay & Nachmansohn, 1938] for which it was postulated by Adrian as early as in 1933 [Adrian, 1933]. There is now evidence that at the central synapses too the concentration of choline esterase is as high as required by the theory of a transmission by acetylcholine. From the experiments of Feldberg & Gaddum it can be calculated that 3×10^6 molecules of acetylcholine are liberated by one single nerve impulse at one single synapse in the superior cervical ganglion of the cat. If we assume the same amount liberated at one single central synapse, and if we assume the same density of cells and synapses as in the sympathetic ganglion, it results that about 10^{13} molecules of acetylcholine could be liberated simultaneously in the thalamus or cerebellum of a fowl, for example. Each of these parts of the brain is capable of hydrolysing about 10^{14} molecules during 1 msec. Even assuming a density of cells several times as high as in the ganglion, the concentration of choline esterase is as high as necessary for removing an adequate amount during the refractory period.

Two other facts may be quoted in favour of a functional significance of choline esterase in the central nervous system, indicating the same role of acetylcholine at the central synapses as in the peripheral nerve endings: (1) There exists not only a big difference of the enzyme concentration between grey and white matter, but in the grey matter too the enzyme is not evenly distributed: it is accumulated at the synapses or their neighbourhood. (2) There is a remarkable parallelism in the tissues of chicken embryos between the development of synapses and the concentration of choline esterase. In brain in which the density of cells

[1] This work was made possible by a grant from the Ella Sachs-Plotz Foundation.

and synapses rises considerably during the last days before birth, the concentration of the enzyme rises at the same time to high values. The $Q_{CH.E.}$ (mg. of acetylcholine hydrolysed during 1 hr. by 100 mg. of tissue) is 10 four days before birth and 20 at birth. It rises to 25 during the first week and is at this time already as high as in the adult animal [Nachmansohn, 1938]. The amount of acetylcholine, which can be hydrolysed by the whole brain at birth, weighing about 800 mg., is 160 mg. in 60 min. After 15 days the brain weighs about 12–1300 mg. and is capable of hydrolysing about 300 mg. in 1 hr.

If the interpretation given of this increase of the enzyme in the brain of chicken embryos is exact, the value of $Q_{CH.E.}$ in brain of mammals, like rats, rabbits and cats, should be relatively low at birth, and the increase should be more important during the first weeks after birth than in chicken brain. For it is known that in these mammals the density of synapses rises particularly during the first weeks after birth.

In the table are given some figures obtained with brain of rats and rabbits. The increase of the enzyme concentration is considerable indeed during the first weeks after birth and, as expected, more important than in chicken brain.

Concentration of choline esterase in the brain of rats and rabbits during the first weeks after birth. $Q_{CH.E.}$ = mg. of acetylcholine hydrolysed by 100 mg. of tissue in 60 min. T = mg. of acetylcholine hydrolysed by the whole organ or part in 60 min.

	New-born*			10 days			20 days		
	Weight mg.	$Q_{CH.E.}$	T	Weight mg.	$Q_{CH.E.}$	T	Weight mg.	$Q_{CH.E.}$	T
Rat, whole brain	204	1·9	3·9	619	3·6	22·2	935	7·2	67·5
Rabbit:									
Whole brain	1350	6·6	90	3183	7·2	229	4419	11·0	488
Telencephalon	778	4·2	33	1924	5·4	104	2556	8·9	228
Diencephalon	277	9·1	25	430	10·2	44	722	15·5	112
Cerebellum	84	5·4	5	297	6·8	20	546	9·0	49
Corp. quadrigemina	60	8·2	5	130	13·3	17	126	20·6	26
Corp. quadrig. ant.	—	—	—	95	14·0	13	83	24·7	21
Corp. quadrig. post.	—	—	—	35	12·5	4·4	43	12·5	5·5
Bulb. olfact.	—	—	—	65	2·9	2	82	4·1	3·4

* The rabbit taken for this column was 2 days old.

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Energy liberation and "viscosity" in muscle. By A. V. Hill.
(*From the Department of Physiology, University College, London*)

Improved myothermic apparatus has made it possible to establish the following relations during a maintained tetanus (English frog's sartorius, winter, 0° C.):

(A) During shortening heat is liberated in excess of that in an isometric contraction and work is done. The extra heat is independent of load and speed, and equal to ax g.cm., where x is the amount of shortening (cm.) and a is a constant of the order of 400 g. wt. per sq. cm. of muscle cross-section.

(B) The *rate* of total extra energy (heat + work) liberation increases linearly with decreasing load (P), and equals $b(P_0 - P)$, where P_0 is the full isometric tension and b a constant of the order of 0·35 of the muscle length per second.

(C) Since heat $= ax$ and work $= Px$, the rate of energy liberation is $(P+a)\,dx/dt$. Equating this to $b(P_0 - P)$, we find

$$(P+a)(v+b) = b(P_0+a). \qquad \text{.....(1)}$$

This relates the speed of shortening ($v = dx/dt$) to the load (P), and can be tested experimentally without any thermal measurements. It is easily verified and a and b agree with the values given above. Fenn's curves [1935] relating speed and force are of this general type.

The term "muscle viscosity" expresses the fact that the force exerted is less the greater the speed of shortening. Fenn [1935, etc.] has long insisted that this apparent viscosity is really due to an adjustment of the energy output to the "work of shortening" [see also Hill, 1931, p. 121]. The new findings show that this view is correct. The rate of energy liberation ($= b(P_0 - P)$) is presumably determined by a kind of potential difference between the chemical store (P_0) and the contractile mechanism (P): if the load is great the rate of energy liberation, and therefore the speed of shortening, must be small: if the speed is great the energy is used largely to supply the heat of shortening and the load must be small.

The heat (ax) of shortening might be due to a constant internal frictional force a. In that case the extra energy would all be liberated as work, some of it appearing externally, the rest as heat in overcoming the internal resistance. Experiments, however, in which a muscle was stretched (slowly) have shown that the extra heat is now negative, which

cannot be explained by a frictional force. It seems that the process of shortening as such is associated with a proportional liberation of heat.

Equation (1) shows that for negative rates of shortening, i.e. for stretching, P should be greater than P_0. The fact is well known. For a speed of stretch equal to b the force should be infinite. Actually the contractile system of the muscle "gives" for forces not much greater than isometric, and the work done is turned into heat, as in a wire stressed beyond its elastic limit. For slow stretches P is not too great, and the work done on the muscle does not reappear as heat.

If we suppose [Gasser & Hill, 1924] that the contractile part of a muscle is in series with a purely elastic part, and that the active state is set up very rapidly after a stimulus, equation (1) can be used to calculate the form of the isometric contraction. We assume that the contractile part shortens at a decreasing rate under an increasing force, stretching the elastic part gradually, until the full isometric force is developed. In equation (1) we put $P=kx$ and $v=dx/dt$ and obtain by integration

$$\log_e \frac{1}{1-P/P_0} - \frac{P/P_0}{1+a/P_0} = \frac{bt}{(1+a/P_0)C}, \qquad \text{.....(2)}$$

where C is the amount of elastic stretch. Inserting known values of a and b and putting $C=$ (say) 10 p.c. of the length of the muscle, the calculated agrees closely with the observed isometric contraction. Moreover, equation (2) tells us that if C is made greater (e.g. by using an elastic connexion to the lever) the development of tension will be proportionally slower. This is easily verified by experiment. The form of the "isometric" contraction does not represent at all the rate at which the active state of a muscle develops: that is much more rapid: it depends simply on the speed at which the contractile part can stretch the elastic part.

For an average representation of the isometric response to a tetanus, of an English frog's sartorius at 0° C., it is sufficient to put a/P_0 equal to its mean value, viz. 0·25, b to 0·35 l/sec. and C to 0·1 l (l = muscle length). Equation (2) then becomes

$$\log_{10} \frac{1}{1-P/P_0} - 0{\cdot}35\,P/P_0 = 1{\cdot}22\,t.$$

For an accurate comparison the stimulus should be maximal and of rather low frequency.

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Eseriniform and curariform actions of certain onium salts.
By GRACE BRISCOE. (*From London School of Medicine for Women*)

Hofmann [1903], using the gastrocnemius of the rabbit with indirect stimulation, showed that curarine depresses responses to slow rates more than responses to high rates of stimulation. On the other hand, eserine depresses responses to high rates more than responses to slow rates [Briscoe, 1936]. This latter finding has been confirmed by Bacq & Brown [1937]. I am indebted to Prof. A. V. Hill for the suggestion that it would be of interest to test the reactions of the quaternary ammonium salts to different rates of stimulation. Dr Ing kindly supplied me with four specimens of onium salts and these have been tested on the quadriceps of the cat by intravenous injection of molar solutions. Records have been made with isotonic and isometric levers with similar results.

Two of the salts, tetramethyl ammonium iodide and phenyl trimethyl ammonium iodide, produce myograms resembling those of eserine in that the responses to fast rates are more depressed than the responses to slow rates. In consequence the normal difference in grading disappears, the fast rate responses become equal to or, more usually, smaller than the slow rate responses (see Fig. 1A). These salts act with great rapidity (10–20 sec.); with subparalytic doses recovery is usually complete in 2–3 min. They are antagonistic to curarine in that a given dose produces less depression after a small dose of curarine. The other two salts, strychnine methiodide and octyl quinolinium iodide, produce myograms like curarine, responses to all rates being reduced and normal grading preserved (Fig. 1B). Their effect is additive to that of curarine; a given dose produces greater depression after partial curarization.

The question arises as to whether these differences in action are due to chemical structure or size of cations.

Raventos [1937], using the rectus abdominis of the frog, found that the lower members of the series Me3NR are antagonized by Me3NOct and curare, and that the action of Me4N is antagonized both by Pr4N and But4N.

The series Me3NR has been tested on the quadriceps, but no sharp change has been found between Me3NOct and the lower members either in their myograms or reaction to curare. There are, however, indications that a change may occur higher up the series. Owing to the difficulty of obtaining the higher members and their relative insolubility this series has not been further explored.

A sharp change has been observed in the series Me4N to But4N. Pr4N and But4N both give myograms of the curarine type (Fig. 1B), their depressant action is strongly potentiated by a small dose of curarine, and they antagonize the depression caused by eserine if given by doses too small to affect normal muscle. On the other hand, Me4N behaves like

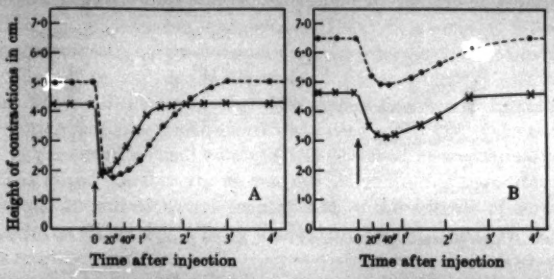

Fig. 1. Quadriceps. Broken line, responses to fast rate at 150 per sec. Unbroken line, responses to slow rate, 30 per sec. A. Eserine type of depression illustrated by effect of Me 4NI; 0·5 c.c. per kg. 5 millimolar injected at arrow. Normal grading lost for period of depression. B. Curarine type of depression. Effect of Pr 4NI, 1·0 c.c. per kg. 10 millimolar. Normal grading is preserved.

eserine, its depressant action is potentiated by eserine and it is antagonized by curarine. The intermediate member Et4N, known to be anomalous, does not produce with comparable doses transient depression of muscle responses.

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Amine oxidase and ephedrine. By H. Blaschko.
(*From the Physiological Laboratory, Cambridge*)

Phenyl*iso*propylamine and its derivatives (e.g. ephedrine) are not oxidized in the presence of amine oxidase, whereas phenylethylamine and its derivatives (e.g. tyramine, adrenaline) are oxidized [Blaschko *et al.* 1937]. The same applies to the aliphatic amines: only those which contain an amino group attached to a terminal carbon atom are oxidized. *N*-butylamine and *iso*-butylamine are oxidized, *sec*-butylamine which has

the amino group attached to the penultimate C-atom of the chain is not oxidized.

Ephedrine acts as an inhibitor of amine oxidase. It shares this property with other derivatives of phenyl*iso*propylamine. The inhibitory effect of a number of those compounds on the oxidation of *l*-*p*-sympatol (Boehringer) in extracts of guinea-pigs' liver is shown below.

Substance	No. of experiments	Percentage inhibition
l-Ephedrine	14	49
d-Ephedrine (I.G. Farbenindustrie)	8	30
dl-Ephedrine	10	40
d-pseudo-Ephedrine	6	32
Suprifen (I.G. Farbenindustrie)	6	44
Veritol (Knoll A.-G.)	5	93

I wish to thank Messrs C. H. Boehringer Sohn, Messrs Knoll A.-G., and the I.G. Farbenindustrie for giving me a number of substances.

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Accessibility of substrate to carbonic anhydrase in red blood corpuscles. By V. H. Booth. (*From the Department of Physiology, Cambridge*)

Meldrum & Roughton [1933] found that, as a rule, dilute suspensions of unlaked corpuscles showed little or no catalytic effect on the rate of evolution of CO_2 from a buffer solution, although the lysed corpuscles showed high carbonic anhydrase activity. Roughton [1935] pointed out that a necessary condition for the active functioning of the enzyme inside the corpuscles was that HCO_3' must enter, exchanging with Cl'. Hence the extent of catalysis is limited by available Cl'. This is negligible in dilute corpuscle suspensions. For the present work Meldrum & Roughton's manometric technique was modified to allow the use of a more concentrated suspension of corpuscles. No enhancement of the rate of CO_2 evolution from phosphate buffer was observed provided there was no laking of the corpuscles, thus showing that Roughton's explanation, though indicating an essential condition, is not alone adequate to account for the facts. There were many reasons for believing that there had been no changes in the permeability of the corpuscle membrane *in vitro* of a magnitude relevant to the present problem, e.g. Dirken & Mook [1931]

mixed red blood corpuscles rapidly with an equal volume of serum previously saturated with CO_2 and found that the HCO_3'—CO_2 equilibria were established in less than 1 sec. Investigation disclosed a possible explanation for the lack of effect with the manometric technique, as follows. During the time (about 10 min.) between measuring the corpuscles into the buffer and beginning the manometric observation the Cl' inside the corpuscles exchanges with HPO_4'' from outside. Phosphate diffuses across the corpuscle membrane too slowly for rapid exchange with bicarbonate, and, as the corpuscle Cl' is now exhausted, no catalysis occurs. The conditions were therefore altered to prevent exhaustion of the chloride and experiments were done both with CO_2 uptake and output. In each case a small catalysis was observed at the *beginning* of a run. The initial (catalysed) rate was the maximum the apparatus could record and its duration was proportional to the amount of corpuscles used. Hence, Cl' no longer being the limiting factor, some other capacity effect must be operative.

The second accessibility factor is doubtless the H^+ which is required for conversion of HCO_3' to H_2CO_3 or vice versa. These ionic reactions can occur in the external buffer solution, but inside the corpuscles *in vitro* they depend upon the buffer capacity of hæmoglobin. When this is exhausted catalysis ceases. *In vivo* additional buffering is provided by the simultaneous oxygenation or reduction of hæmoglobin.

The conclusions are established that, *in vitro*, (1) corpuscles, although rich in carbonic anhydrase, can only perform a given small amount of catalysis in each direction per cycle, and (2) the cellular enzyme cannot directly accelerate reactions occurring in the plasma.

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The "slow potentials" associated with excitation and inhibition in the excised eye. By RAGNAR GRANIT and P. O. THERMAN. (*From the Institute of Physiology, Helsingfors*)

From the point of view of recording potentials in the central nervous system it is instructive to consider the result of an analysis of "slow potentials" in the nervous structure composed of an enucleated, opened

frog's eye with its optic nerve. The recording apparatus is a cathode ray oscillograph and a directly coupled amplifier. The four leads shown to the left in the figure are employed. Differentiation of the various components of the complex response is achieved by means of two known effects of potassium. A drop of an 0·5 p.c. solution of KCl on the retina (i) removes the discharge in the optic nerve before the retinal response is diminished, and then (ii) attacks the retinal response to illumination so that the positive component P II is selectively removed and the electroretinogram only consists of the negative component P III.

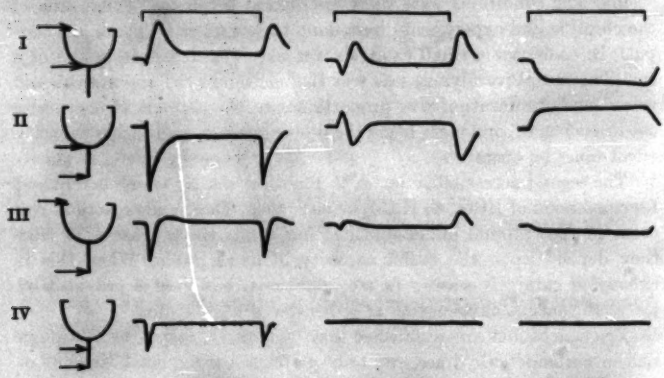

Fig. 1.

Lead I is the standard lead for recording the electroretinogram of an excised opened eye. Lead IV is the one generally employed for picking up the impulses in the optic nerve. For all leads positivity of upper electrode relative to lower electrode is plotted upwards in the figure. The complex normal responses in leads II and III are largely due to negativity along the nerve. The large negative deflexions diminish when the nerve is pinched near the bulb and disappear when, "soon after potassium", the nerve ceases to respond. Lead II then, as is to be expected, gives an inverted electroretinogram, lead III a small normal electroretinogram. In this phase lead I may give normal responses but generally they are changed in the direction illustrated. When finally the retinal response, "later after potassium", consists of a pure negative P III, this is inverted in lead II and normal but very small in lead III. The return to the base

line of the negative P III reproduces the properties of the off-effect when wave-length, intensity or state of adaptation is varied.

The experiments show that two processes of opposite sign can be localized to the retina. When the latter is removed from the eye it soon dies, but it lives long enough to show that with the positive component P II the sensory epithelium is *negative* relative to the ganglion layer, whereas with the negative component P III the free end of the sense cells is *positive* relative to the ganglions (as the results of Fig. 1 suggested).

It has previously been shown that the components P II and P III are associated with respectively excitation and inhibition in the optic nerve. With the aid of a double-ray oscillograph and a pair of condenser coupled amplifiers simultaneous records with leads I and IV have now been taken. (These enable a more accurate estimation of the moments of onset of excitation or inhibition in retina and nerve than previously has been possible.) For this purpose it has been necessary to use the off-effect where either process can be selectively elicited in the retina. It is found that a deflexion in the direction of P II begins in the retina before the nerve discharges. The onset of inhibition is preceded by or coincides with the appearance of the component P III in the retina. Such results would not be expected if P II and P III were after-potentials. The retinal potentials do not show the pharmacological properties characterizing after-potentials.

We do not know whether either component *causes* excitation or inhibition or both. The evidence available only shows them to be genuine slow potentials in the retina, regularly associated with excitation (P II) and inhibition (P III) in the manner described. They seem to spread electrotonically, as is best shown by the fact that rod and cone potentials are mutually refractory though elicited by different receptors (differentiation by the aid of wave-length).

Osmotic pressures and albumin-globulin ratios of sera of normal and immunized rabbits. By G. S. and M. E. Adair. (*From the Departments of Physiology and Pathology, Cambridge*)

Govaerts [1927] reported that the osmotic pressure of human serum was a function of the albumin-globulin ratio and gave a formula for the calculation of the osmotic pressure of serum. Wells *et al.* [1933] stated that Govaert's formula was unreliable and concluded that the ratio p/C, where p = osmotic pressure and C = concentration of total protein

is independent of the globulin concentration and depends only on the concentration of albumin. Their observations indicate that albumin alters the osmotic pressure of globulin and they note that the molecular weight of globulin in serum might differ from that of purified globulin.

We have compared the formula of Govaerts and that of Wells with measurements of the osmotic pressures of sera of normal rabbits and of rabbits which have been injected with crystalline egg albumin. The presence of antibody causes a considerable diminution in the albumin-globulin ratio. Heidelberger & Pedersen [1937] have shown that the molecular weight of globulin in immunized rabbits is very close to that of the principal normal globulin component. Measurements were made at 0° C. by the method of Adair [1925]. In a few experiments an ultrafiltrate of serum or antiserum was used as dialysate, but in all the experiments recorded in Figs. 1 and 2 the dialysate was a Sørensen buffer mixture $M/30$ $Na_2HPO_4 + M/30$ KH_2PO_4. Protein concentrations were calculated from nitrogen determinations, assuming that albumin contains 15·6 p.c. N and globulin 15·13 p.c. N.

For the purpose of comparing the work of Govaerts and of Wells *et al.* with our measurements, all pressures given by these authors have been reduced to mm. Hg. at 0° C., and protein concentrations have been recalculated using the nitrogen factors given above.

In Fig. 1 the ordinate $\pi = p/C$, where p = osmotic pressure and C = g. total protein per ml. solution. The percentage of the total protein in the form of albumin is plotted as abscissa. The broken line has been calculated from the formula of Govaerts. Our observations showed that π depends not only on the albumin-globulin ratio but also on the total protein concentration C. The three lines marked $C = 8$, $C = 6$ and $C = 4$ have been derived from a series of measurements made at different protein concentrations.

In Fig. 2 values of π have been plotted against the concentration of albumin in g. per 100 ml. solution, as suggested by Wells *et al.* Their formula, represented by the broken line WY, appears to agree with our measurements more closely than the formula of Govaerts. Whereas Wells *et al.* studied a large number of sera showing a wide variation of albumin-globulin ratios and obtained one value for each serum at approximately the protein concentration existing in the body, we have examined only a small number of sera but have determined in each case a pressure-concentration curve. Since our measurements indicate that the relationship between π and the concentration of albumin is not determined by a single line but by a series of lines, with slightly higher

values for π in solutions in which the albumin-globulin ratio is high, they do not confirm the hypothesis of Wells *et al.* "that the specific pressure of the globulin in any serum is the same as that of the albumin with which it is associated".

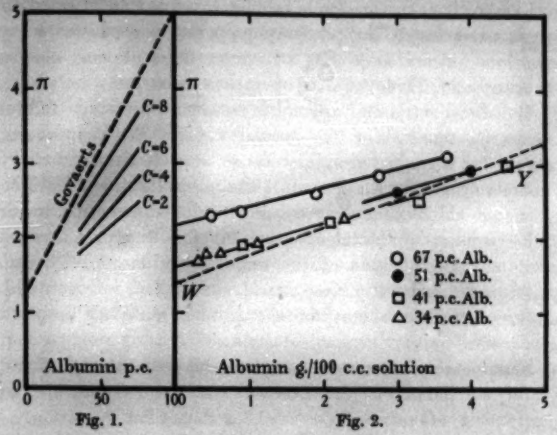

Fig. 1. Ordinates $\pi = p/C$. Abscissæ: albumin p.c. expressed as percentage of total protein.

Fig. 2. Open circles, normal rabbit serum, albumin 67 p.c. of total protein. Closed circles, squares and triangles, immunized rabbit sera.

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Experimental renal insufficiency following intrarenal arterial injection of posterior pituitary extract. By S. H. Liu[1] and R. L. Noble.[2] (*From the Courtauld Institute of Biochemistry, Middlesex Hospital, London*)

It has been reported by Dodds *et al.* [1934] that renal damage was produced in rabbits after subcutaneous injection of large doses of posterior pituitary extract. Byrom [1937] observed similar changes in rats and described the pathological findings in detail. During some observations

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on water balance in rabbits following subcutaneous pituitary extract administration, two animals died with marked retention of blood urea and non-protein nitrogen, while other animals consistently showed casts in their urine.

In the following experiments on rabbits, extracts were injected directly into the renal artery. The pituitary preparation used was a purified posterior lobe extract containing 10 pressor units per mg. dissolved in 0·01 *N* acetic acid. Three types of operative procedure were adopted:

(1) Unilateral intrarenal arterial injection at one stage followed in 2–4 weeks by removal of the normal kidney. With approximately 10 units per kg. the injected kidney was so severely damaged that death with uræmia occurred on removal of the normal kidney. With 5 units per kg. enough kidney function remained to enable the animal to survive.

(2) Preliminary unilateral nephrectomy followed in 1–2 weeks by intrarenal arterial injection of the remaining kidney. With doses of 1–5 units per kg. a definite retention of non-protein nitrogen and urea was observed, although most of the animals survived despite renal impairment.

(3) Nephrectomy on one side simultaneous with the injection of the renal artery on the other side. Doses as small as 1–3 units per kg. resulted in anuria, uræmia and death in 3–4 days after operation.

Urinary findings after intrarenal arterial injection consisted of suppression of urine and appearance of red blood cells and hyaline and granular casts. Albuminuria was usually slight, rarely exceeding 0·05 p.c. Blood showed, apart from non-protein nitrogen and urea retention, slight or moderate anæmia in all the cases. Blood pressure of the ear artery determined by means of Grant's capsule revealed no consistent changes except in two cases where definite elevation occurred. Histological examination of the injected kidneys indicated that glomerular and tubular damage was associated with smaller doses, while areas of infarction were produced by larger doses.

Control injection of the oxytocic fraction of the posterior pituitary (pitocin 3 units per kg.) gave entirely negative findings.

In conclusion, renal damage and functional derangement may be readily elicited in rabbits by the injection into the renal artery of relatively small doses of the pressor principle of the posterior pituitary gland.

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The influence of testosterone propionate on the changes induced in monkeys by gonadotrophic hormone. By S. ZUCKERMAN. (*From the Department of Human Anatomy, Oxford*)

Testosterone propionate suppresses the menstrual cycle in rhesus monkeys and completely stops cyclical ovarian changes [Zuckerman, 1937]. Related observations on rats [Parkes & Zuckerman, 1938] suggest that this inhibition is an indirect effect through the pituitary. The following experiments were accordingly carried out on monkeys in order to discover whether or not testosterone propionate can inhibit the effect of injected follicle-stimulating hormone (F.S.H.).

Twelve rhesus monkeys whose body weights varied between 2·17 and 4·06 kg., and most of whom were post-pubertal, were studied. The F.S.H. preparation used was "Antex", assayed at 8000 mouse units per g. Antex has only a follicle stimulating effect on monkeys [Engle & Hamburger, 1935]. After the preliminary removal of a control ovary, four monkeys were given Antex alone for periods varying between 11 and 21 days. In two monkeys one ovary was removed during and the other at the end of the period of Antex injections. The remaining six monkeys were first given an initial course of Antex injections (8–14 days), at the end of which one ovary was removed. The Antex was then continued, together with daily injections of testosterone propionate, for periods varying between 8 and 14 days. In most experiments the daily dose of Antex was 500 M.U., while the amount of testosterone propionate varied in different experiments between 25 and 200 mg. daily.

The control experiments in which F.S.H. alone was given showed that the ovaries may increase up to ten times their initial size during a period of 20 days of Antex injections. The response varies in different animals, and in one case it was found that the ovary removed at the end of the course of injections weighed less and was smaller than the ovary removed in the middle of the period of treatment. Of the animals that were given testosterone propionate in addition to Antex in the second half of the experiment, one gave an equivocal result, two showed a decrease, while three showed an increase in the size of the ovary. In one of the latter three animals the ovary *in situ* at the end of the experiment weighed 7·8084 g., which is anything from 15 to 40 times the size of the normal ovary. This animal was given 1·7 g. of testosterone propionate in the last ten days of its experimental treatment. As in the other animals, the enlargement was due to relatively enormous follicular cysts.

These experiments show that testosterone propionate cannot inhibit circulating F.S.H., and they suggest that its power to inhibit the follicle stimulating effects of the pituitary in normal monkeys is due to a change which it causes in the anterior lobe of the pituitary itself. The experiments do not imply that testosterone propionate would necessarily be ineffective in decreasing the size of natural cystic ovarian follicles which owe their presence to excessive F.S.H. action, and which persist because of their inability to rupture.

Study of the uteri in the twelve animals used in these experiments shows that the proliferative effects of the œstrogenic hormone produced by the Antex-stimulated ovaries was partly inhibited in those animals which received testosterone propionate.

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The ovarian responses of hypophysectomized rats and X-ray sterilized mice to testosterone propionate. By A. S. Parkes and S. Zuckerman. (*From the National Institute for Research, Hampstead, and the Department of Human Anatomy, Oxford*)

The uterine cycle in rats and in monkeys can be suppressed by the administration of male hormone. In rats the inhibition of the cycle is associated with a prolonged phase of luteinization, as is evidenced by the presence of new corpora lutea and by the capacity of the uterus to respond to trauma by decidual formation [McKeown & Zuckerman, 1937]. The following experiments suggest that the ovarian response to the injection of testosterone propionate in these animals is dependent on the presence of the pituitary.

Three normally cyclic female rats were hypophysectomized and were then injected with 0·2 mg. of testosterone propionate daily for 10 days. Injections were started 9 days after the operations. On the sixth day of each course of injections a thread was tied longitudinally into the lumen of each uterus. Autopsies were performed on the eleventh day.

The ovaries were much smaller than normal but each contained several apparently recent corpora lutea. Their presence cannot, however, be taken to indicate that they had been formed after the start of the injections, for corpora lutea degenerate much more slowly after hypo-

physectomy than they do in normal animals, and the corpora may, therefore, have been formed before the removal of the pituitary.

No uterus shows any trace of a decidual reaction. The uterine horns were small, and in no case does their histological character suggest progestational changes.

Another three hypophysectomized animals were treated in the same way except that 1 mg. of progesterone was substituted for the daily injection of 0·2 mg. of testosterone propionate. In normal animals this treatment also leads to a phase of luteinization. No decidual reactions were observed in the present experiments. Slight progestational changes are present in one uterus and these were probably due to the injected progesterone.

On the basis of current views of gonadal-hypophyseal relationships, it would therefore follow that testosterone propionate, when injected into female rats, stimulates the pituitary to liberate its luteinizing hormone. Certain discrepancies were met when this theory was tested.

Study of the ovaries and of the progestational and decidual reactions of the uteri of ten mice showed that the mature mouse responds in the same way to the injection of testosterone propionate as the rat. It was found, however (seven experiments), that the ovaries of X-ray sterilized mice do not luteinize as a result of testosterone propionate as they do in response to luteinizing pituitary extracts. Further experiments on seventeen animals also showed that the ovaries of the normal pubertal mouse do not usually luteinize in response to either the injection or implantation of testosterone propionate, in spite of the presence of numerous moderate sized follicles. This latter result may, however, be related to the well-known fact that the follicles of the immature mouse do not normally respond readily to luteinizing hormone, a fact which is attributed to insufficient preliminary stimulation by autogenous follicle-stimulating hormone.

Two of the three hypophysectomized rats referred to above, and which were injected with testosterone propionate, came into œstrus during the course of treatment. Histological study shows that the vagina of one was cornified at the time of autopsy. Five of seven other hypophysectomized rats which were also injected with testosterone propionate (but whose uteri were not traumatized) also came into œstrus during the course of treatment. The possibility that the œstrus was a response to the trauma of frequent vaginal examination was excluded in control experiments on hypophysectomized rats which were not injected with male hormone.

Testosterone propionate has no œstrogenic action on the vagina of the normal mature rat, and its œstrogenic powers on the hypohpysectomized animal may be attributed, among other possibilities, either to the lower threshold to œstrogens which such animals appear to have [Zuckerman, 1938] or to the possibility that testosterone is converted in the hypophysectomized animal to some related compound which has stronger œstrogenic powers.

It may be noted that mucification of the vaginal epithelium occurs in X-ray sterilized mice injected with testosterone propionate, as it does in normal mature female mice, and also in mature female rats.

We wish to express our best thanks to Dr R. L. Noble for his kindness in providing us with hypophysectomized rats.

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Blood-sugar raising action of extracts from urine of non-pregnant, pregnant and lactating women. By S. C. Werch.
(*From the Department of Physiology, Trinity College, and National Maternity Hospital, Dublin*)

It is well known that the anterior pituitary produces a blood-sugar raising substance (reviews by Collip & Russell), and that similar substances have been found in the blood stream and in the urine. The fact that the anterior pituitary is specially active during pregnancy suggests that this "glycotropic" action may also be increased.

Extracts were prepared from 24 hr. urine specimens of healthy women before, during and after pregnancy. The method of preparation used was that described by Houssay & Biossotti, and adapted by Werch & Altschuler in their study on urine from diabetic and non-diabetic patients. The final product was brought in each case to a uniform volume of 50 c.c., and rabbits were used for intravenous injection of 2 c.c. portions. Blood samples for quantitative sugar determinations were taken before and after injection.

Previous observations by Werch & Altschuler indicated that urine from healthy individuals produced little or no rise of blood sugar in dogs and rabbits. Further studies during the present investigation fully confirm these conclusions.

Preparations made from urine of healthy women in the first three months of pregnancy gave no consistent rise of blood sugar. The substance, therefore, would seem to be absent, or present only in small amounts, in the early months of pregnancy.

Extracts prepared from the urine of healthy women in the last three months of pregnancy gave a definite rise of blood sugar in every case. The average rise in one series of six tests was 24 mg. p.c.

Extracts prepared from the urine of healthy women in the first two weeks after delivery gave even greater blood sugar rises. The average rise in one series of experiments was 55 mg. p.c.

An extract was prepared from the urine of one diabetic patient in the last month of pregnancy. This product tested on three rabbits produced blood sugar rises of 76, 99 and 117 mg. p.c. (maximum readings in each experiment). Further investigations on diabetic patients are in progress.

Preliminary tests carried out to evaluate the properties of the products used in the above work indicate that they are probably the same as the yields studied by the investigators referred to above. It seems possible that the products used in these experiments may be similar to those described by Anselmino & Hoffman, Funk, Katzman & Doisy, and Harrow. This question is also being investigated at present.

It would be of interest to know if blood-sugar raising substances obtained from urine are similar to the more pure substance isolated by Young and used by Himsworth & McNair Scott.

It is suggested that the glycosurias often present during pregnancy, and sometimes explained as being due to temporary lowering of renal threshold, may also be caused by increase in production of the glycotropic factor of the anterior pituitary.

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Blood flow through cat's tongue during contraction. By G. A. MILLIKAN. (*From the Department of Physiology, Cambridge*)

Perhaps the simplest method of studying changes of blood flow and oxygen metabolism in a mammalian muscle is by direct spectroscopic observation through the tongue of the anæsthetized animal. This can be done on cats, without any operation at all, if a powerful beam of white light is passed through the tip of the tongue, while it is loosely held between two parallel glass plates, the emergent light being analysed either by eye with a spectroscope or photoelectrically with colour filters. Due to the relatively small amount of muscle hæmoglobin in the cat's tongue and the large amount of blood, most of the changes observed concern the blood and not the muscle pigment (in contrast to the cat's soleus, where the proportions are reversed).

During rest, strong and dark oxyhæmoglobin bands are seen, but when the tongue is tetanized by 50 cycle induction shocks directly applied through electrodes at its tip and base, the oxy-bands are replaced by a *much* fainter reduced hæmoglobin band, in from 3 to 6 sec., the process being reversed as soon as the tongue is allowed to relax. The change in intensity of the spectral bands shows that much blood is being forced out of the tongue during the onset of contraction, at the same time that oxygen is being withdrawn from that remaining. If tetanus is continued, there is a sudden reappearance of the oxyhæmoglobin bands, nearly as dark as in the resting state, at about the 10th second, showing that an inrush of blood has taken place, bringing with it enough oxygen to supply the immediate metabolic needs of the active muscle and leave the blood still quite well oxygenated. The full initial intensity of the oxyhæmoglobin bands reappears almost as soon as the stimulus is discontinued in exactly the same way as after a short tetanus.

The method can be controlled by clamping off the circulation (rubber band around the tongue) and by the injection of cyanide, as has previously been done for the cat's soleus preparation [Millikan, 1937]. The general nature of the phenomena is unmistakable whether observed visually or recorded photoelectrically, but the quantitative interpretation of the photoelectric records is complicated by the fact that we are dealing with two phenomena (changes in hæmoglobin concentration and changes in oxygen saturation), which cannot be completely separated by the methods used.

These findings are in accord with those which have just been reported by Kramer for dog's gastrocnemius, whose results confirmed those of Anrep for short tetani and those of Rein for long ones.

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Direct determination of fœtal oxygen consumption. By M. F. Mason, J. A. Kennedy and J. Barcroft. (*From the Department of Physiology, Cambridge*)

The amount of oxygen used by the fœtus per g. per min. has been estimated by several workers, and systematically over a considerable portion of fœtal life by Barcroft *et al.* [1934]. In view of the degree of surgical interference involved in the method used by these authors it seemed desirable to try some quite different technique.

(1) The umbilical cord is tied.

(2) Within about $\frac{3}{4}$ min. successive samples of blood are taken from the carotid artery or the femoral. The oxygen content of these is measured, and at first, this plotted against time gives a straight line (see Fig. 1). If

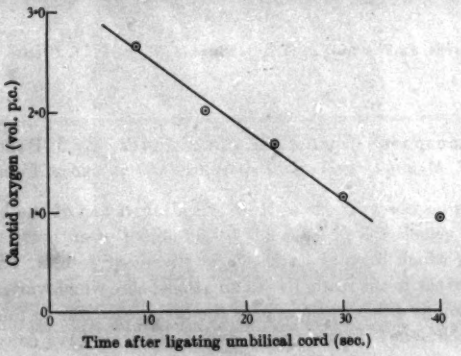

Fig. 1.

the amount of blood in the fœtus is known, the oxygen consumption per g. per sec. of the fœtus is

$$\frac{-dO_2}{t} \times \frac{BV}{W},$$

where $-dO_2$ is the loss of oxygen by the arterial blood during a time t during the time that the loss is linear. BV is the blood volume and W is the weight in grams.

If the oxygen difference between the arterial and venous blood is determined just before the tying of the cord, the volume of fœtal blood which passes through cotyledons may be calculated, and correspondingly if the oxygen difference in the maternal blood entering and leaving the cotyledons be known, the amount of maternal blood which traverses the cotyledons may be calculated approximately.

Example:

Oxygen lost by arterial blood in 21 sec. = 1·50 vol. p.c.

Blood volume, measured after delivery = 340 c.c.

Weight of fœtus = 3650 g.

O_2 difference between umbilical vein and umbilical artery = 5·46 vol. p.c.

O_2 difference between carotid and uterine venules = 6·13 vol. p.c.

Therefore:

Fœtal oxygen consumption = 0·004 c.c. per g. per min.

Blood flow through the umbilical cord = 267 c.c. per min.

Lower limit of maternal blood flow through the cotyledons = 238 c.c. per min.

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The atmosphere in which the fœtus lives. By J. Barcroft and M. F. Mason. (*From the Department of Physiology, Cambridge*)

Numerous observations of the oxygen saturation of the blood in the umbilical vessels have been made by a number of authors, and also by ourselves, which have given the most divergent results. The question arose, does the fœtus really live in an atmosphere which varies greatly in composition at different times or is the divergence due to faulty experimental technique? In a series of experiments we have convinced ourselves that between the 75th and 140th days of fœtal life we observed the following conditions: (1) The fœtus was not taken from the uterus. (2) A general anæsthetic was not given, spinal and local being used. (3) The position of the uterus was only interfered with to the minimal extent.

Under these conditions the distribution of figures for oxygen saturation was as follows:

Saturation of blood with oxygen ...	90 and over	89–80	79–70	69–60	59–50	49–40	Mean
Number of cases: Umbilical vein	5	3	2	—	—	—	87
Umbilical artery	—	—	—	3	6	1	58
Uterine venule	—	—	3	1	2	—	66

The above figures indicate an atmosphere of much higher oxygen saturation than has hitherto usually been regarded as existing.

Nerve fibres of the odontogenetic layer of the dentine. By W. Lewinsky and D. Stewart. (*From the Department of Anatomy, Manchester University*)

Tiegs [1938] has recently described a very rich ramification of fine nerve fibres in the odontogenetic zone of the dentine. These were seen particularly well in sections cut tangentially or semi-tangentially along

Fig. 1. Ramification of nerve fibres (*N*) in the odontogenetic tissue (*O*) which is lightly stained. The black masses are portions of the coarsely calcified zone (*C*) which separates the odontogenetic layer from the fully calcified dentine.

the margin of the pulp cavity. The fibres were extremely fine and many of them terminated in small bulbous swellings (*boutons terminaux*) in relation with the Tomes fibres in the dentinal canal. On account of their fineness and the different depths of their ramification, Tiegs was unable

to photograph them in their entire extent. We have prepared similar sections and have also seen free ramification of nerve fibres. These fibres, however, differ in several features from those described by Tiegs. They are much thicker. They do not appear to end in special nerve-end organs and have no particular relationship to the fibres of Tomes. We also had no difficulty in photographing, as will be seen by the photomicrograph which accompanies this paper. It would appear, therefore, that there are two types of nerve fibres ramifying in the odontogenetic layer, the fine fibres of Tiegs with special nerve endings, and the thicker fibres described by ourselves, which do not have such an ending. Tojoda [1934] and Lewinsky & Stewart [1936] have described both thick and thin fibres in the odontogenetic layer of the dentine. The thin fibres enter the mouths of the dentinal canals, while the thick fibres run in the ground substance. It is now suggested that these thick fibres were merely portions of the ramifications described in this present paper.

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Rhabdonecrosis (retinitis pigmentosa) in the rat. By Margherita C. Bourne, Dorothy R. Campbell and Katharine Tansley. (*From the Department of Physiology and Biochemistry, University College, London, and the Birmingham Eye Hospital*)

In the course of an investigation on the crystalline lens the authors acquired a stock of rats bearing an inherited retinal degeneration often associated with cataract.

The microscopic appearance of the retina during certain stages of the disease is almost indistinguishable from that found in the few retinæ that have been examined in cases of human retinitis pigmentosa, and there seems to be little doubt that the two conditions are the same. Both are inherited as a Mendelian recessive character.

The earliest sign of abnormality so far recognized in the diseased rat retinæ occurs when the rats are about 21 days old, the time at which the retina reaches its final stage of differentiation. At this age scattered degenerations are seen among the nuclei of the visual cells (most of which are rods in the rat), and in the course of the next few weeks these degenerations spread until the whole of the outer nuclear layer of the retina

is dead. The death of the rod nuclei is accompanied by a slow progressive breaking up of the rods themselves, so that by the time the rat is 12 weeks old the outer part of the retina has disappeared.

This primary change resulting from the initial death of the visual cell nuclei is followed during the life of the rat by profound secondary changes such as extensive fibrosis of the retina, alteration of the structure of the pigment epithelium and fibrous adhesions between the retina and the choroid. Very often a typical posterior cortical cataract also follows.

In trying to elucidate the underlying cause of this disease the first question we have to answer is—why do the visual cells die? At present we do not know the answer although we have some isolated pieces of information which we hope may be helpful in finding it.

Although the lesion is hereditary it does not appear to be due to a failure in development. The retina of an affected rat follows the normal course of development, as far as one can trace it, until the very last stage of differentiation—that of the visual cell nuclei—has been completed. It is after the final stage has been reached and not until then that the visual cells begin to die. It seems, therefore, that the inherited weakness from which these highly specialized cells suffer does not interfere with their ability to survive fœtal conditions or even to differentiate, but does make it impossible for them to meet the requirements of adult life.

A simple universal amplifier. By BRYAN H. C. MATTHEWS.
(*From the Physiological Laboratory, Cambridge*)

Direct coupled amplifiers are more convenient and reliable than condenser coupled amplifiers for recording any changes of more than 0·1 sec. duration. A simple D.C. amplifier has recently been described by Forbes & Grass [1937], but it gives sufficent gain only for use with a high sensitivity instrument, such as a string galvanometer. The amplifier here described, while comparatively simple, can give as much amplification as can at present be used with any form of oscillograph and is suitable for use in most kinds of physiological recording.

This amplifier has been evolved from that previously described [Matthews, 1935] in the course of work on the slow potential changes of the spinal cord, and while it contains no special novelty it combines a number of desirable features achieved by the simplest available methods. No output stage is shown, as the design of this depends on the type of oscillograph used.

To set the amplifier (Fig. 1) P_1 is moved to zero amplification and P_2 set so that the light from the recording instrument is on the centre mark of the camera. P_1 is then turned up and the zero thenceforth controlled only by R. The tapping T is so placed that the anode current of the two valves AA_1 is approximately equal: it may need readjustment after some months' use.

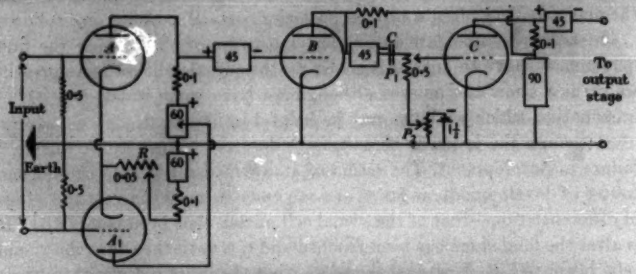

Fig. 1. Circuit diagram of amplifier. (Cathode heater circuits have been omitted.) Battery voltages and resistances in megohms are marked.

(1) Creep is minimized by the use of a balanced input stage [Matthews, 1934] with a high resistance common to the cathodes; the merits of the latter were pointed out to me by Dr Tonnies (verbal communication) and fully confirmed in use [see Schmitt, 1938]. The amplifier is controlled by the potential between the two grids. The use of paired valves greatly reduces slow changes of zero, which rarely exceed 10 microvolts per min. Creep can be completely avoided by the arrangement described by Buchthal & Nielsen [1936], but the complication necessary is considerable. The noise level is approximately 2 microvolts on open circuit.

(2) Time lags will occur unless the capacity to earth of the coupling batteries is kept low. Small deaf aid batteries (Drydex), supported clear of screens, etc. have been found very satisfactory: no current is drawn from them and their life is consequently long. The calibration (Fig. 2) shows that the distortions arising thus are very small. Milliameters, if inserted in the anode circuits, by their large inductance introduce very serious errors, and if used must be subsequently short circuited.

(3) The amplification can be altered by the potentiometer R without producing any movement of the zero.

(4) For many purposes direct coupling is unnecessary and condensers can be introduced at C, so that creep is eliminated; but a milliameter

must then be used in the anode circuit of valve *B* to check the setting of R_2 and, if necessary, then short circuited (see (2) above).

(5) The overall amplification is about × 60,000, using Mazda AC/2 HL or Mullard 904 v valves, which with an output stage is adequate for many purposes, but it may be increased up to about × 1,000,000, which is more than can ever be usefully employed, by using H.F. pentodes

Fig. 2. *a*, Calibration of amplifier with triode valves. 150 μV. *b*, Calibration with pentode valves in two stages *B* and *C*, 50 p.c. full amplification; 20 μV.; 10,000 ohms between grids and earth. Time marker 0·02 sec. intervals.

(Mullard SP 4) at *C* or *B* and *C*; holders for either type may be wired in parallel. Calibration of these two alternatives is shown in Fig. 2. The instrument used is the author's oscillograph, fed by two pentodes in parallel operated from the A.C. mains. For calibration between both grids and earth the deflexion produced is 1 p.c. that of calibration grid-grid.

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A blood-volume method using a blue dye and photocell. By J. A. KENNEDY and G. A. MILLIKAN. (*From the Department of Physiology, Cambridge*)

This is a dye-injection method using photocell colorimetry, and requiring only 0·3 c.c. of plasma, thus making it suitable for small animals as well as large.

The dye used is T–1824 or Evans Blue. Its blue colour can be measured photoelectrically without serious error even in the presence of considerable quantities of hæmoglobin, errors due to hæmolysis being thus eliminated. The dye is further well fitted for blood-volume work by virtue of its nontoxicity, its slow disappearance rate in the body, its stability, and its freedom from absorption by cellular constituents of the blood.

The dye concentration is determined by its light absorption, using a simple photocell colorimeter, consisting of a car headlamp bulb, a red filter, Wratten No. 29, a trough of 0·1 c.c. capacity and thickness of 1 mm., a barrier type photocell and a pointer galvanometer. It has been found empirically that under the experimental conditions employed, Beer's law is obeyed, i.e. the dye concentration is proportional to the negative logarithm of the galvanometer deflexion.

Standardization is made by measurement of a known dilution of dye in saline containing 20 p.c. serum, which gives the same light absorption as an equal concentration of dye made up in whole serum, and it is remarkably constant for sera from different individuals of the same species.

The advantages of this method, as compared with the usual procedures using vital red dyes and visual colorimetry, appear to be:

(1) Avoidance of rapid disappearance rate.

(2) Freedom from error due to hæmolysis and the presence of serum pigments.

(3) Freedom from subjective judgments and eye fatigue in colour matching.

The apparent accuracy of the method is about 3 p.c.

An improved apparatus for stimulation by electromagnetic induction. By H. W. Newman. (*From the National Hospital, Queen Square, London*)

Previous methods [Chaffee & Light, 1934; Fender, 1936] have been modified in two respects. First, high frequency (450 kc.) current is used to energize the primary coil surrounding the animal cage, with resultant greatly increased induction incidental to the increased rate of charge of flux within the field, and ability to use much smaller currents. This high frequency current will not stimulate, but must be rectified by a copper oxide rectifier in the secondary unit, the resulting stimulating current

depending for its wave form on the modulation of the primary current. Second, instead of using three primary coils, with the difficulties of energizing them in sequence, a single primary coil is used with a secondary implanted unit consisting of three coils mutually at right angles, each with its individual rectifying unit, but all connected to the two stimulating electrodes. By this expedient the output at the electrode is maintained relatively constant regardless of the position of the animal.

The advantages of the apparatus are simplicity, low current consumption, and the ability to use any wave form desired, while the size of the animal cage may be greatly increased over the limits practicable with previous methods.

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A case of amputation of leg, 90 days before birth. By D. H. Barron and J. Barcroft. (*From the Departments of Physiology and Anatomy, Cambridge*)

The experiment with which this demonstration deals is one where the spinal cord of the sheep fœtus was cut on the 50th day of fœtal life and in the fœtus allowed to continue growth. The section was at the level of the 4th cervical. The fœtus was born by Cæsarian section on the 140th day (approximately normal term) and therefore survived in the uterus for 90 days after the section.

At birth it weighed 1900 g., which was rather light but not abnormal. With the exception of the right leg, which was accidentally amputated during the operation, the lamb was normally proportioned, and the development of the bones, etc., was perfect.

The injury to the 4th cervical region made respiration impossible; therefore the lamb only survived a few minutes. It was extremely hyperactive and kicking violently so long as the placental circulation was intact.

Special interest attaches to this forelimb, the portion of which distal to the elbow joint was amputated. The skeleton demonstrated shows:

Scapula reduced in size, spine about one-third of normal, distal epiphysis reduced in about the same degree.

Humerus, same length as on left side but much reduced in every other respect. The bone a little rarified.

The spinal cord in the segments distributed to the limb; both the dorsal and ventral roots are about one-third the size of the normal side. There is a corresponding reduction in the size of the same half of the cord. Both the grey matter and the white are reduced by about a third. Most of the reduction in the white matter is in the posterior columns.

PROCEEDINGS
OF THE
PHYSIOLOGICAL SOCIETY
4 *June* 1938

Increase of resistance to oxygen want in rats on a carrot diet.

By J. Argyll Campbell. (*From the National Institute for Medical Research, London*)

White rats (80 g.) are exposed to barometric pressure 240–250 mm. Hg (O_2 pressure = 7 p.c. of 1 atm.) in the small decompression chamber at Messrs Siebe Gorman & Co., S.E. 1, taking 5 min. to reach this low pressure. At the end of the 2nd min. the pressure reaches 480 mm. Hg, at the end of the 3rd min. 380 mm. Hg, at the end of the 4th min. 280 mm. Hg and at the end of the 5th min. 250 mm. Hg. When the temperature of the chamber is kept at 20–25° C. all the rats usually survive 30 min. exposure, but when the temperature is 33° C., as a rule all of the rats on normal diet die within about 10 min. at 245 mm. Hg. The latter conditions therefore supply a simple and ready means for testing effects of various agents upon powers of resistance to oxygen want; the test is obviously severe and should therefore be efficient. This method of testing effects of various oxygen pressures with the aid of variation of external temperature was demonstrated to the Society on 12 December 1936 [Campbell, 1937, 1938].

With this technique most of the usual vitamins, hormones, cardiac stimulants and various other substances and conditions of diet have been investigated and most of them give no aid whatever towards resisting the above degree of oxygen want. When testing for vitamin A, a pure carrot diet was liberally employed and the carrots, whether raw or boiled for 15 min., were found to give definite help (see Table I). After 5 days on

Table I. Resistance of rats to barometric pressure 245 mm. Hg. $O_2 = 7$ p.c. of 1 atm.; $T = 33°$ C.

Condition of animals	No. of animals at start	Survivors at 5 min. intervals					
		10 min.	15 min.	20 min.	25 min.	30 min.	35 min.
Carrot diet for 5 days	21	21	21	21	20	18	15
Starvation for 1–2 days	25	10	4	3	3	2	2

this diet the rats lose about 10 p.c. of their weight, but they resist and survive exposure to 245 mm. Hg pressure for 30 min. in place of the usual less than 10 min. for the rats on normal diet. The effect is not due to pure vitamin A nor to carotene. It is not due to loss of weight nor starvation (see Table I); starvation for 1–2 days protects slightly but does not give such definite aid and rats 80 g. do not tolerate starvation beyond 48 hr.; loss of weight is 10–20 p.c. in this time. On the carrot diet the glycogen in the liver falls to 0·5–0·7 p.c. (kindly estimated by my colleague Mr H. P. Marks and his assistant, A. Spratt), which is also the figure given for liver glycogen in rats starved for 1–2 days [Evans *et al.* 1931]. Thus low glycogen content of the liver is not responsible for the relieving effect of the carrot diet. It does not appear to be connected with acid-base equilibrium as neither an acid nor an alkaline diet gives any help. The possibility of a detoxicating substance has been considered but xanthin [Neale & Winter, 1938] gives no relief as it does with chloroform poisoning. Other substances of interest which have also given negative results are nicotinic acid, cocaine, lactoflavin (I am indebted to Dr O. Guggenheim, Basle, for a sample), adenosine, carbon dioxide and ammonium chloride.

My colleague, Dr C. Rimington, has suggested that glutamine may be responsible, and with his co-operation experiments are being conducted with various fractions from the carrot substance.

The experiments have been fully controlled. It is not merely a temperature effect since the rats tolerate and survive 35 min. at 375 mm. Hg with temperature 33° C.

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The action of urea on the small intestine of the rabbit. By S. Lazarus. (*Introduced by* G. H. Bell.) (*From the Institute of Physiology, University of Glasgow*)

Urea is a normal constituent of the blood and tissue fluids and has generally been accepted as a waste product having little or no pharmacological action. Rona & Neukirch [1912] reported that urea had no effect on the rabbit jejunum, *in vitro*, even when a concentration of 0·5–1 p.c. was reached.

In the present experiments, segments of rabbit jejunum were suspended in a bath containing 100 c.c. of Tyrode-Bayliss at 37° C. The addition of 10 mg. of urea in 2 c.c. of fluid produced, in a considerable proportion of cases, an increase in the amplitude of the segmental movements and a slight rise in the tone of the bowel. Larger doses of urea, 20 and 40 mg., produced similar effects. Still larger doses of urea, 200–400 mg. in 2 c.c. of fluid, produced a fall in tone and a diminution of segmental movements. This inhibition was transient but could be demonstrated in every case. The factor determining the change in the

Fig. 1. Successive tracings from a single experiment showing the effects of increasing doses of urea on eserinized rabbit jejunum. Interval between tracings: 2 min. The bowel was not washed between additions of urea.

motor activity of the bowel segment appeared to be the sudden increase in the concentration of urea in the bath because ample movements could be recorded when concentrations of more than 1 p.c. were attained.

The motor effects of urea were found to be accentuated if the bowel segment was first treated with eserine salicylate (1 : 2,500,000), and then washed. Successive additions of 40, 100 and 200 mg. of urea to the bath resulted in successive increases in the tone and segmental movements of the bowel. An initial inhibition preceded the motor effects of the two larger doses (Fig. 1). In spite of eserinization the addition of 400 mg. of urea to the bath fluid resulted in a marked, although transient, fall in tone and amplitude of segmental movements (Fig. 1).

At present only a tentative explanation of these findings can be offered. Acetylcholine is present in the bowel muscle and can be demonstrated in the bath fluid containing an isolated segment of bowel [Lazarus, unpublished]. It would appear that a chemical reaction occurs in the bowel muscle which results in the formation of acetylcholine. The action

of urea, in the lower doses, may result by its replacing the acetyl radicle in this reaction. It is interesting to note in this respect that carbaminoylcholine, which is analogous to the urea-choline compound that may be formed, has a somewhat greater activity than acetylcholine itself on the isolated segment of rabbit intestine [Noll, 1932]. The inhibition of the destruction of the urea-choline compound by eserine may account for the greater motor effects observed after eserinization. It is possible that the inhibition produced by large doses of urea is an osmotic effect. These doses, in any case, result in concentrations of urea which are greater than those ever met with in body fluids.

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The acetylcholine content of the nerves of warm-blooded animals. By O. Loewi and H. Hellauer. (*From the Pharmacological Institute, Graz*)

In all the efferent nerves acetylcholine is present; it is to be noted, however, that the proportion present in preganglionic sympathetic fibres is about six times as great as in postganglionic fibres. Purely sensory nerves, of which the optic nerve was taken as an example, and purely sensory nerve fibres, as found in the dorsal roots running centrally from the spinal ganglia, do not contain even traces of acetylcholine. The importance of the latter observation for the question of the significance of acetylcholine in the mechanism of the propagation of impulses in nerves will be obvious.

Galactose-1-phosphate in the liver during galactose assimilation. By H. W. Kosterlitz. (*From the Physiology Department, Marischal College, Aberdeen*)

In a previous paper [Kosterlitz, 1937] evidence was brought forward which suggested the presence of a galactosephosphate in the liver during galactose assimilation. Analysis by fermentation showed that the non-nitrogenous phosphoric esters isolated from the livers of five groups of galactose-fed rabbits contained between 19 and 37 p.c. of galactosephosphate, 11 and 28 p.c. of glucosephosphate, besides unidentified esters. The galactosephosphate appeared to be non-reducing, and it was tentatively suggested to be a galactose-1-phosphate.

Since then α (?)-galactose-1-phosphate has been synthetized by a method similar to that used by Cori *et al.* [1937] for the synthesis of α (?)-glucose-1-phosphate. The $[\alpha]_D$ of the dibrucine salt in pyridine is −32·2° and that of the secondary barium salt in water +81·3°. After 5 min. hydrolysis in 0·09 *N* H_2SO_4 at 100° 92 p.c. of the ester was split; heating for a further 10 min. did not increase the hydrolysis. The liberated galactose was identified as the methylphenyl hydrazone. The ester was non-reducing. Treatment with *S. Ludwigii* set free 95 p.c. of the galactose present which is completely fermented by subsequent treatment with galactose-adapted *S. cerevisiae* Frohberg.

Thirty-eight rabbits have been fed with galactose and the phosphoric esters isolated in the manner described in the previous paper. A product (1·75 g.) containing 30 p.c. of galactosephosphate and 16 p.c. of glucose-phosphate was obtained. At least 9–10 mg. of galactose were present as the phosphoric ester in 100 g. of liver. Attempts to isolate the pure ester were unsuccessful, fractional crystallization as the brucine salt being impossible owing to the small yield. By fractional precipitation with aqueous alcohol a product was obtained which contained 45 p.c. of galactosephosphate; subsequent precipitation with neutral lead acetate yielded a filtrate from which a substance containing 53 p.c. of the ester was isolated.

The view that the naturally occurring ester is a galactose-1-phosphate is supported by the following facts: (1) In both naturally occurring and synthetic esters the galactose was liberated by treatment with *S. Ludwigii* which caused an increase in reduction, and then fermented by galactose-adapted *S. cerevisiae* Frohberg. (2) Both esters were hydrolysed in 10 min. by 0·09 *N* H_2SO_4 at 100°. The ratio of liberated galactose to inorganic P was in good agreement with the theoretical value of 5·8 to 1. The galactose contents of both esters as calculated from the increase in reduction after acid hydrolysis agreed with those obtained from the analysis by fermentation. This can be so only if the esters are non-reducing. (3) That the increase in reduction after acid hydrolysis for 10 min. was actually due to the splitting of the galactosephosphate present in the isolated material was proved by the formation of the galactose methylphenyl hydrazone and by the estimation of the liberated galactose after its separation from the unhydrolysed non-galactose esters.

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Closing of ductus arteriosus. By A. E. Barclay, J. Barcroft, D. H. Barron and K. J. Franklin. (*From the Nuffield Institute for Medical Research, Oxford, and the Physiology Department, Cambridge*)

Radiological and X-ray cinematographic studies have been made on practically full-term lambs delivered by Caesarean section. The results indicate that the ductus begins to close within a few seconds from delivery and that it is often completely closed, so far as the transmission of blood is concerned, within 1 min. The research will be published *in extenso* in the *British Journal of Radiology*.

Recording of pulse rate during exercise. By G. H. Bell and J. A. C. Knox. (*From the Institute of Physiology, University of Glasgow*)

It is well known that it is not possible to obtain an electrocardiogram while the subject is moving about, and similar difficulties obtain in attempting to record the radial pulse during exercise. For these reasons pulse rates in exercise tests are still counted by palpation, with its inevitable errors.

Fig. 1. Effect of exercise on the pulse rate. Upper trace, time in seconds. Middle trace, electrocardiogram (coupling condensers, 0·05 μF., grid leaks 1 megohm). Lower trace, respiration. At the moment indicated by the first arrow the subject stood up and stepped five times up two steps (each 10 in. high) and down again. The second arrow indicates the end of the exercise.

We have found that if the time constant of the intervalve coupling of the electrocardiograph amplifier is reduced very considerably below the normal value, then the effect of skeletal muscle activity is negligible.

The slow *T* and *P* waves of the electrocardiogram are, of course, not reproduced on the tracing, but this is no disadvantage when records of pulse rate only are required. The tracing reproduced here was obtained on smoked paper by coupling the amplifier to a suitable writing galvanometer.

The advantages of this method are that the subjective factor is eliminated, and also that a very much closer analysis of the response of the heart to exercise is possible.

Pulse tracings obtained by this method during various forms of exercise will be demonstrated.

PROCEEDINGS
OF THE
PHYSIOLOGICAL SOCIETY
2 *July* 1938

The respiratory response to the excitation of a sensory nerve.

By Wm. H. Wilson and M. Hammouda. (*From the Physiological Department, Faculty of Medicine, Cairo*)

We have described [1935] the effects of stimulating the central end of the cervical vagus, below a spot cooled to 8° C. or lower, as an acceleration of the breathing in place of the inhibition normally occurring.

Gessell, *et al.* [1937] have suggested that the results we obtained, and ascribed to the excitation of fibres coming from the lungs carrying impulses the function of which is to augment the excitability of the respiratory centre, are due to the excitation of sensory fibres in the vagus, not necessarily coming from the lungs, and have the same explanation as the respiratory response to stimulation of any sensory nerve, effects which are commonly ascribed to pain.

With a view to examining the validity of this criticism a comparison has been made between the effects of exciting the vagus and a somatic sensory nerve (sciatic, peroneal and saphenous).

The normal effect of stimulating the central end of the sciatic, in the anæsthetized animal, is a great increase in the rate and usually a considerable increase in depth of breathing. With light anæsthesia tonic expansion of the thorax, with irregular, rapid and shallow respiration, occurs.

We find that these effects on respiration are absent in the decerebrate animal, while the augmentor effect of exciting the vagus below a cooled spot is equally well shown in the decerebrate as in the anæsthetized preparation.

We have assumed, further, that if the augmentor fibres in the vagus were comparable to those present in a sensory nerve, the stimulation of which gives rise to a reflex acceleration of the breathing, the effect of cooling would be similar.

A number of observations has been made on the three nerves mentioned above and on the vagus in the same animal, in which the nerves were cooled to below 8° C. and faradized below the cooled area. In all cases it was found that while excitation above the cooled spot caused the usual respiratory response, little if any change in the breathing occurred when the nerve was faradized distally, indicating that the sensory fibres in the sciatic, the excitation of which leads to reflex acceleration of the breathing, are blocked at a temperature of 8° C. On the other hand, excitation of the vagus under identical temperature and other conditions, produces, as we have frequently shown, a well-marked increase in the rate of respiration.

Furthermore, we find that, while (with the open chest) excitation of the thoracic vagus below the root of the lung gives no respiratory response, excitation of a pulmonary branch or of the vagus above the root of the lung (in each case with the cervical vagus partially blocked by cooling) leads to a well-marked acceleration of the breathing.

It may be concluded that the respiratory accelerator effects of exciting the vagus are not analogous to the respiratory response to excitation of a sensory nerve, either in their central origin or in their functional significance.

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Diaphragmatic spasm caused by curarine. By J. W. THORNTON.
(*From the Department of Physiology, Bristol University*)

West [1938] investigated the respiratory failure caused by curarine, and in that publication kindly acknowledged collaboration with me in some experiments on isolated lungs. He concluded that the primary factor in this failure is diaphragmatic "loss of tone", and that constriction of the bronchi by the drug is an important accessory factor. The latter conclusion seemed to me questionable both in the light of my observations and also after analysis of West's own results. My evidence will be published in full later, as work is still proceeding, but during the course of recent experiments a new factor has been found that has a probable bearing on the subject. This is that curarine causes diaphragmatic spasm before the onset of the expected paralysis, and furthermore,

this occurs with doses smaller than those required to produce constriction of the bronchi.

I am using a method that gives simultaneous records of bronchial tonus and diaphragm movements in cats (anæthetized with chloralose). This method has been developed from one previously described [Thornton, 1937] which gives simultaneous records of systemic blood pressure and of bronchial tonus from one lobe of a lung that has its natural blood supply. The new addition is a separate record of diaphragm movement, and, since the respiratory reflexes are intact, although the chest is open and the lungs are artificially ventilated, it is possible to stop and start the diaphragmatic rhythm at will by altering the amount of ventilation. This is done by adjusting the screw clip on the side tube of the tracheal cannula, and the best adjustment is one which gives a moderate excursion of the diaphragm.

Drugs are injected into a leg vein, and in the case of curarine the maximum concentration in the blood has been calculated from the dose on the assumption that the minimum blood volume is $\frac{1}{15}$ of the body weight. Results are tabulated:

Curarine concentration in the blood	1/150,000	1/35,000	1/20,000
Diaphragm:			
Duration of spasm	2 min.	40 sec.	20 sec.
Time until paralysed	6½ min.	52 sec.	30 sec.
Broncho-constriction	Absent	Absent	Absent

In only two experiments has broncho-constriction been obtained with concentrations of this order, and they were earlier experiments before the diaphragm record was added; the dose in each case was 10 mg., which gave respective blood concentrations of 1/20,000 and 1/25,000. Usually three or four times this dose is necessary to constrict the bronchi in this type of experiment.

Before the injection of curarine various controls are used to test the sensitivity of the preparations—such as intravenous adrenaline and acetylcholine and faradic stimulation of the vagus in the neck. If the same tests are repeated after the curarine it is found that all their recorded reactions are considerably depressed, that is to say both the bronchial and blood-pressure reactions, and this suggests that curarine may depress transmission at autonomic nerve endings as well as at those of voluntary muscle. There is, however, not yet enough evidence in my results to point with certainty to the site of the depression.

It is possible that the spasm of the diaphragm here recorded may play a part in the respiratory embarrassment which precedes the fatal

asphyxia in curarized animals. This possibility is strengthened, in my opinion, by watching animals curarized, when the signs of effort seen suggest a spasm of the respiratory muscles and particularly the diaphragm.

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Nerve degeneration and bone hypertrophy induced in young animals by diet. By EDWARD MELLANBY

Some years ago I described destruction of the eighth nerve in young dogs by diets deficient in vitamin A and rich in cereals [Mellanby, 1933]. This destructive change affects the ganglion cells (spiral and Scarpa) of both branches of the nerve and often results in complete deafness of the animals [Mellanby, 1937]. In addition to the nerve degenerative changes, there is overgrowth of bone of the labyrinthine capsule. Bone in the modiolus of the cochlear hypertrophies and causes pressure on the fibres of the eighth nerve; there is also overgrowth of the periosteal bone of the labyrinth capsule at the exit of the internal auditory meatus. This lengthens the distance from the cochlear or vestibule to the brain and so stretches the nerve. Examination of the labyrinthine capsule and its nerves under these conditions suggests that the bone overgrowth is responsible for the nerve degeneration.

On the other hand, nerve degeneration has been shown to be very widespread when young animals are brought up on these diets. Thus many of the afferent nerves of the body, both cranial and somatic, degenerate, as also do ascending tracts in the central nervous system [Mellanby, 1931, 1934, 1935]. The question must be faced whether all these instances of nerve degeneration are due to bone overgrowth and the pressure and stretching of the nerves thus produced. No definite answer can yet be given to this question, but it is undoubted that the bone overgrowth is not confined to the labyrinthine capsule. Examination of the skulls of these animals, for instance, shows other bone abnormalities and it is clear that other nerves are also squeezed by bone overgrowth at the various foramina. This certainly applies to the trigeminal and the facial and possibly to other nerves. Examination of

the spinal column for bony overgrowth is being made to see whether the resulting squeezing and stretching may explain degeneration of the somatic nerves.

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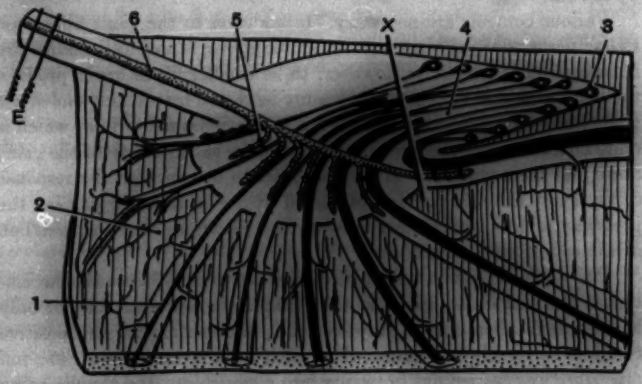
Synaptic transmission in the absence of nerve cell bodies. By J. Z. Young. (*From the Department of Zoology and Comparative Anatomy, Oxford*)

In his well-known "*Carcinus* experiment" of 1897 Bethe showed that normal reflex movements of the antenna of the crab could take place after all of the cell bodies had been removed from the T-shaped efferent neurons. The experiment was so difficult, however, that it was only fully carried out on three animals and apparently it has never been successfully repeated. In the squid, *Loligo*, the conditions allow a comparable experiment to be more easily made. The large nerve fibres (1)



which produce the rapid contraction of the mantle muscles, (2) arise as syncytia by fusion of the processes of cells, (3) which lie in a special giant fibre lobe, (4) of the stellate ganglion [see Young, 1936]. Collateral

branches (5) of the large motor fibres make synaptic contacts *outside the giant fibre lobe* with branches of the presynaptic fibre (6) coming from the central nervous system. It is therefore possible by the cut *X* to sever the lobe, including all of the cell bodies of the giant fibres, while leaving the synaptic contacts undamaged.

The operation can be rapidly performed without anæsthesia by inserting a blade from the dorsal side through the transparent mantle muscles. After severance of the giant fibre lobe on each side in this way the animals were still capable of shooting violently through the water by quick contractions of the mantle, indicating that the giant fibres were activated by impulses from the central nervous system even after their cell bodies had been removed. Unfortunately they do not stand operation well, and the longest survival obtained has been only 30 min. With special precautions longer survivals might be obtained, but the proof that conduction across synapses is possible in the absence of cell bodies can be given as well, if not better, by an acute experiment. Condensor discharges applied through electrodes at *E* produce each a single impulse in the presynaptic fibre, which sets up a single impulse in the postsynaptic fibre, and this in turn produces a twitch of the muscles. After removal of the giant fibre lobe the response can still be obtained, except from the hinder part of the mantle whose giant fibre is severed by the cut.

Various controls are necessary. In addition to the giant fibre each stellar nerve also contains smaller motor fibres, whose cell bodies are not removed by the cut. However, these smaller fibres never produce the quick maximal twitch, as can readily be shown by pricking one of the giant fibres [Young, 1938]. Presence of the maximal twitch, which is quite characteristic, therefore clearly indicates the integrity of the giant fibres and their synapses. Finally, in order to be sure that the response of the giant fibres is not due to spread of the stimulating current the presynaptic nerve is pinched between the electrodes and the ganglion at the end of each experiment.

The whole experiment must be carried out rapidly, since the circulation is not well maintained and the synapses remain excitable for only half an hour after death. However, this allows time for the positive result to be readily demonstrated on both sides of the animal. The full procedure was carried out in twenty-two experiments, the ganglia being afterwards fixed and sectioned for verification of the level of the cut. The results prove decisively that as Bethe claimed, and as might perhaps be expected, synaptic excitation of one nerve fibre by another does not

depend on the presence of the nucleus, Golgi bodies, Nissl substance or any other substances present in the nerve cell body.

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Acetylcholine in the cerebro-spinal fluid. By H. M. Adam, R. A. McKail, S. Obrador and W. C. Wilson. (*From the Departments of Surgery and Pharmacology, University of Edinburgh*)

Feldberg & Schriever [1936] found that acetylcholine (ACh.) appeared in the cerebro-spinal fluid (c.s.f.) of dogs after the intravenous injection of eserine; the ACh. content of the c.s.f. was increased by intravenous adrenaline and by asphyxia but not by central vagus stimulation. We have tried to trace the source of this ACh. with slightly modified methods.

In cats and dogs the ventricular system was "perfused" with eserinized Locke solution (1 in 2×10^5) run through from lateral ventricle to subcerebellar cistern at a pressure of from 2 to 10 cm. water and at a rate of about 1 c.c. in 2 min. Provided that no pronounced rise in blood pressure or intracranial tension was produced the perfusion could be maintained at a constant rate and pressure. ACh. appeared in the effluent fluid, and its concentration remained constant for several hours. In some experiments eserine was infused intravenously throughout at a slow rate; the ACh. concentration in the perfusate was then higher but steady.

During perfusion the central ends of the following nerves were stimulated for 10 min. periods: vagus, trigeminal branches, sciatic, cervical sympathetic, splanchnic, carotid sinus nerves and the spinal cord cut in the cervical region. In all cases stimulation produced no significant rise in ACh. concentration. A breathing mixture of 7 p.c. carbon dioxide in air was also ineffective.

Stimulation of different parts of the brain was then tried. Stimulation, by a 50 cycle alternating current, of the motor cortex and of the cerebellar cortex gave negative results. In the case of the hypothalamus, however, the results were positive in seven of fourteen experiments; the concentration of ACh. was approximately doubled. The increase may

have been merely the result of heating, or other local effect, at the electrodes, as in stimulating nerve trunks [Gaddum *et al.* 1937], but, against this, a constant current produced no change in concentration. It is possible that a small localized area in the hypothalamus must be stimulated in order to produce an increase in ACh. concentration. This point is being investigated.

In one other circumstance the ACh. content rose significantly. Perfusion of the ventricles with eserinized Locke solution containing pituitrin (or pitocin or pitressin) in a strength of 10 milliunits per c.c. give a concentration of ACh. about twice as great as that obtained with eserinized Locke solution without pituitrin. With higher dilutions of pituitrin the results were variable. The increase was not due to a change in *p*H of the perfusing fluid or to the action of preservative. In view, however, of the quantity of pituitrin required to produce the increase no special significance can be attached to the observation. Intravenous pituitrin, ventricular perfusion with adrenaline (1 in 10^5), and very slow intravenous infusion of adrenaline (1 in 10^8) did not alter the ACh. concentration.

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Glucose and acetylcholine synthesis in a ganglion. By F. C. MacIntosh. (*From the National Institute for Medical Research, Hampstead*)

Brown & Feldberg [1936] found that when the cat's superior cervical ganglion was perfused with Locke's solution containing glucose and eserine, and subjected to continuous stimulation through the preganglionic nerve, the total quantity of acetylcholine appearing in the effluent greatly exceeded that obtained by extraction of the control ganglion; nevertheless, they observed no depletion of the ACh. store of the perfused ganglion at the end of stimulation. During the stimulation the ACh. in the effluent fell to a steady minimum.

I have repeated these experiments, applying continuous preganglionic stimulation to a ganglion perfused with Locke's solution containing eserine, but without glucose. Under these conditions the ACh. output falls more rapidly to a lower minimum, with depletion of the store in the ganglion; and the transmission of impulses, as indicated by the

contraction of the nictitating membrane, diminishes with the output. When both have fallen to a low level, the addition of glucose to the perfusion fluid regularly brings about an immediate, though not complete, restoration of the peripheral response, with a simultaneous increase of the ACh. output.

It is of interest to compare this observation with that of Mann *et al.* [1938] on the aerobic formation of an ACh. precursor, by brain substance, from choline in the presence of glucose. Dr G. Kahlson and I are testing other sugars and derivatives.

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Precipitin tests and anti-prolactin serum. By W. I. Strangeways. (*National Institute for Medical Research, Hampstead*)

The evidence which is accumulating, relating serological manifestations *in vitro* and "anti-hormone" (Collip) activity of serum produced by the administration of anterior pituitary or other hormone-containing extracts, is, as yet, inconclusive. (See, for example, Bachman [1935], Twombly [1936], Werner [1938], Oudet [1938].)

The present investigations were carried out with anti-prolactin serum produced by the daily administration to rabbits of large doses of relatively purified ox prolactin [Young, 1938]. The injections were continued over a period of some months so that the serum was relatively rich in non-specific precipitins. These were greatly reduced by precipitation of the antiserum with the optimum proportion of normal ox serum, found by the method of Dean & Webb [1926]. This "ox-adsorbed" serum still contained precipitins to prolactin solution which were much reduced after precipitation of the serum with the optimum proportion of this solution.

The anti-prolactin (pigeon crop gland test) activities of the "ox-adsorbed" and "ox- and prolactin-adsorbed" sera were determined. The results are summarized in Table I.

The results show that (1) the precipitate formed with ox serum did not remove any of the activity from an anti-prolactin serum, (2) about two-thirds of this activity was removed by further adsorption with the optimum amount of prolactin solution, and (3) that no activity was lost when the serum was precipitated with one-fifth of this optimum amount.

TABLE I. The influence of specific precipitation on the activity of anti-prolactin serum

Prolactin assays by the method of Rowlands [1937] on groups of five pigeons

Ox prolactin injected daily, mg.	Serum: Type	Serum: Previously precipitated with	Amount injected daily, c.c.	Crop gland response % ± S.D. mean
0	—	—	—	0·25 ± 0·01
5	Normal rabbit	—	0·5	0·98 ± 0·09
5	Anti-prolactin	—	0·5	0·38 ± 0·02
5	Anti-prolactin	Ox serum	0·5	0·39 ± 0·03
5	Anti-prolactin	(1) Ox serum (2) Ox prolactin (O.P.)*	0·5	0·73 ± 0·09
5	Anti-prolactin	(1) Ox serum (2) Ox prolactin (½ O.P.)	0·5	0·33 ± 0·03
5	Normal rabbit	Mixed with amount of prolactin ≡ O.P.	0·5	0·87 ± 0·13

* O.P. = optimum proportion found by the method of Dean & Webb [1926].
½O.P. = ½ optimum proportion found by the method of Dean & Webb [1926].

Material was not available for further biological experiments on the specificity of the reaction but the results suggest that the anti-prolactin activity is specifically precipitated by some antigen contained in the prolactin fraction of a pituitary extract, if not by prolactin itself. Further *in vitro* tests showed that, after precipitation with ox serum, the anti-serum still contained precipitins against sheep prolactin as well as ox prolactin. Preliminary tests also indicated that the *in vitro* activities of a number of different prolactin preparations paralleled their pigeon crop gland activities.

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On the identity of blood monocytes and tissue macrophages; their growth rates *in vitro*. By F. JACOBY. (*From the Department of Physiology, The University, Birmingham*)

Carrel & Ebeling [1926] showed that hen blood monocytes and macrophages derived from tissues other than blood are morphologically indistinguishable when kept *in vitro* under identical conditions, i.e. in similar states of activity. Similarly, tissue culture fibroblasts derived

from different sources do not show any essential morphological differences. However, Parker [1932] states that they can be distinguished according to their different inherent growth energy and he, therefore, speaks of "races" of fibroblasts, a claim which still awaits confirmation. The question arose as to whether in a similar way "monocytes" and "tissue macrophages" have to be considered as different "races"; this is especially important in view of the alleged close relation between the two.

Results of the growth rate of hen blood monocytes *in vitro* have been reported in a previous communication [Jacoby, 1937]. Corresponding experiments have now been performed on "tissue macrophages". These were obtained from fresh heart explants of chick embryos (5–14 days old). Such cultures, when growing in Carrel flasks under heparin plasma feeding, become after some time (10–20 days) surrounded by a wide halo of macrophages which, being very transparent, contrast markedly with the very fatty fibroblast culture which first formed from the explant.[1] The ring of coagulum containing the macrophages was then excised and transferred as a lump into another flask and dealt with in a manner corresponding to the Baker technique which had been used for the blood monocytes. Owing to the weak fibrinolytic power of the macrophages only in a small number of experiments did sufficient cells migrate from the old transplanted clot to form a healthy population attached to the glass bottom of the vessel. Thus the number of successful experiments was limited. After removal of the clot containing the transplanted piece of coagulum a uniform population of macrophages was obtained in this way, and when after a few days at 39° C. it had ceased to multiply it was again fed with serum (1 c.c.) diluted with Tyrode solution and its rate of multiplication was determined. In some experiments this was done by the use of Willmer's serial photographic method giving the percentage of dividing cells per unit time in a given field ($\frac{1}{4}$ mm.^{2}). Photographs were taken every 6 min. for several days. Fig. 1 shows the curve of such an experiment. As with blood monocytes, there was a latent period of about 20 hr. before the onset of mitotic activity (in most experiments it varied between 18 and 22 hr., in one experiment it was even 30 hr.). Then the cells divided actively for a further period of about 20 hr. after which the mitotic activity came gradually down to zero again. The mitotic response was in some experiments somewhat feebler than with blood monocytes. This might be connected with the heparin treatment prior

[1] The macrophages were seen to migrate from the interior of the culture. Their origin within the culture is not known but, clearly, as to their past history they differ widely from the blood monocytes of an adult hen.

to the sojourn in the second flask, because it is known from experiments on fibroblasts that heparin has a growth inhibiting effect [Fischer, 1936]; a point which is under further investigation. In other experiments the whole population present was counted at intervals; the results are

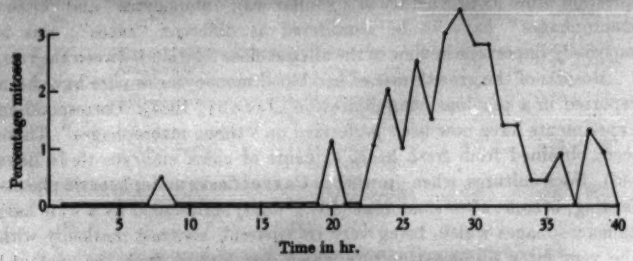

Fig. 1. Flask culture M45/8593 *b*, tissue macrophages, 45 days *in vitro*. At zero hour fed with 1 c.c. 25 p.c. hen serum.

TABLE I

Culture No.	Type of culture	Number of cells present: At start of exp.	After 24 hr.	After 48 hr.	After 72 hr.
8424	B.M.	1016	—	2486	4753
8425	B.M.	241	252	536	696
8574 *b*	T.M.	240	280	—	660
8654 *a*	T.M.	1413	1933	3136	3262

B.M. = blood monocytes. T.M. = tissue macrophages.

given in Table I and compared with figures obtained from blood monocytes cultures. Moreover, such cultures of macrophages living in Carrel flasks attached to the glass and fed with serum could be kept alive and active for about the same length of time as had been possible with blood monocytes, i.e. about 3 months.

The results show that the two cell populations of different origins in regard to their growth rate when living in serum behave very much alike under comparable *in vitro* conditions, thus adding a further common physiological character other than morphological to these two sets of cells increasing the evidence of their identity.

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The appearance of fat in the urine after short occlusion of the renal artery of the dog. By E. B. Verney and Marthe Vogt.[1] (*From the Pharmacology Laboratory, Cambridge*)

While studying the effects on water diuresis and arterial pressure of short occlusions of the renal artery in the dog, we encountered a phenomenon which proved to be as sensitive an indicator of impairment of renal function as the rate of water excretion itself. The "compression unit" employed for occluding the renal artery was that described by Rydin & Verney [1938]. The unit is implanted behind the kidney at a preliminary operation, and its application, in those experiments with which this communication deals, implies denervation of the one kidney and removal of the other. The renal artery lies in a channel drilled in a bakelite block; this channel can be occluded by inflating a thin rubber tube suitably embedded in the block and connected with fine pressure tubing which runs through the uterus to the vagina: here it may be connected, when so desired, with a micrometer-operated syringe filled with the inflating fluid.

Occlusion of the renal artery does not seem to produce any sensation in the dogs, since they neither move nor show any sign of discomfort during the compression. Samaan [1936] and Rydin & Verney [1938] have described the rapid suppression of urine flow when the renal artery is compressed for a short period (3 min.), and the equally rapid and complete recovery of diuresis when the compression is released. If the urine excreted after compression of the artery be analysed, there will be found albumen and a transitorily increased concentration of chloride, provided the compression has lasted sufficiently long: the minimum period for the production of both effects in one of our animals was 1 min. If the compression be shorter neither of these alterations is seen, but the urine becomes cloudy, and the cloudy material accumulates at the surface when the urine is centrifuged. On microscopical examination this cloudiness is found to consist of isotropic fat droplets of various sizes. In the three dogs in which such experiments have been made, occlusion of the renal artery was regularly followed by the occurrence of fat droplets in the urine; these droplets were also present in the absence of any other change detected after longer periods of compression. Occlusions of as little as 5 and 10 sec. duration were sufficient for the excretion of fat provided they also produced an inhibition of water diuresis; this inhibition of flow, however, needed by no means to reach

[1] Alfred Yarrow Research Fellow, Girton College.

complete suppression. An example is given in Table I : it is a record of the results of nine consecutive occlusions of the renal artery carried out at intervals of 10 min. If compression of the same duration were repeated, a similar inhibition of urine flow ensued on each occasion; the amount of excreted fat, however, diminished, but increased again when the periods of compression were lengthened.

TABLE I

No. of occlusion	Duration in seconds	Inhibition of diuresis %	Albumen	Concentration of chloride	Fat
1	10	31	—	Unchanged	Present
2	30	71	—	,,	Less than in 1
3	30	75	—	,,	,,
4	30	51	—	,,	Trace only
5	30	53	—	,,	,,
6	60	100	Trace	85% increase	As in 1
7	60	100	,,	107% ,,	,,
8	60	100	,,	45% ,,	Less than in 1
9	600	100	Large amount	700% ,,	Large amount

Since large amounts of fat are normally present in the descending distal limbs of the proximal convoluted tubules of the dog's kidney, secretion of this fat into the tubules is the probable explanation of the phenomenon. Such a secretion is known occasionally to occur in the dog in infectious disease [Henschen, 1923], and the results of the present experiments can be interpreted as the production of the same effect by renal ischæmia. The decreased production of fat when the obstruction is repeated can hardly be explained by exhaustion of available fat, since longer periods of ischæmia are then effective in increasing the production. The amount of secreted fat varies in different dogs; in the three which we observed, the smallest quantity was produced by a kidney in which a kink in the renal artery was causing a slight and continuous obstruction of blood flow. It is not unlikely that in this animal the resulting chronic ischæmia had depleted the normal tubular fat depots.

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The absorption of anæsthetic agents from the urinary bladder. By R. R. MACINTOSH and C. L. G. PRATT. (*From the University Laboratory of Physiology, Oxford*)

Various drugs have been introduced into the urinary bladder of the cat by means of a hypodermic needle inserted through the abdominal

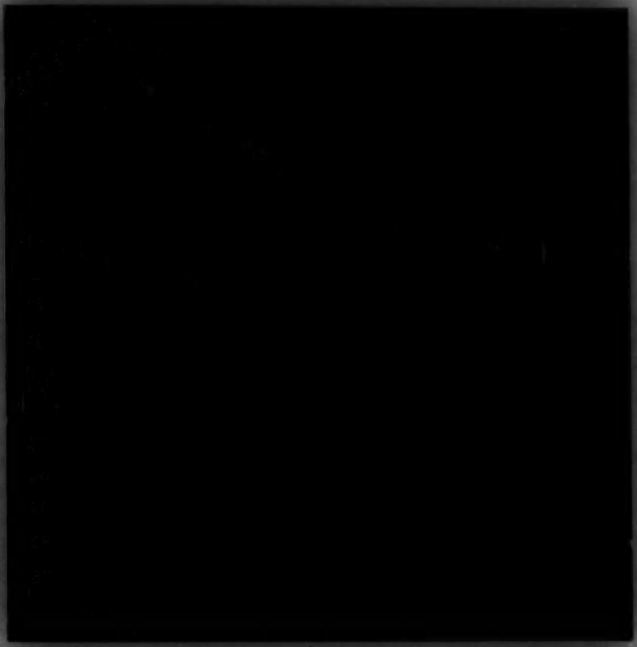

Fig. 1. Effect of intravesical avertin on blood pressure, bladder volume and bladder movements. Chloralosed cat 3·5 kg. Upper record, blood pressure; lower, bladder volume. Bladder pressure constant at 12 cm. H_2O. At arrow, 0·3 g. avertin into bladder.

wall. Cocaine, tribromethanol ("avertin") and a number of barbiturates in common clinical use all produce general as well as local effects when introduced in this way. General anæsthesia may be induced in a cat by placing 0·6 g. tribromethanol in the bladder. This quantity is about 20 times the rectal dose for a 3 kg. cat. Drowsiness and incoordination

supervene within 5 min.; full anæsthesia develops in about 10 min., and, if the bladder is not emptied at once, lasts for 5 or 6 hr. An intravesical dose of 0·3 g. produces some lethargy or even incoordination, but does not bring about anæsthesia.

The anæsthetic dose of cyclohexenylmethyl-*N*-methyl barbituric acid ("evipan") given by this method is 2 g., which is about ten times as great as the intraperitoneal dose for a 3 kg. cat.

It has not been possible to demonstrate any correlation between dosage and body weight, or between dosage and bladder volume at the time of injection.

All the drugs so far tried tend to cause congestion of the bladder epithelium. If the bladder is emptied by manual compression as soon as general anæsthesia is established, untoward effects usually do not follow, but if the drug is left in the bladder for many hours, a hæmorrhagic cystitis may supervene, and this may be fatal.

The process of micturition does not seem to be interfered with after the recovery of consciousness.

The introduction of any of these drugs into the bladder of the cat already under chloralose anæsthesia produces a prompt dilatation of the bladder and diminishes the amplitude of, or even abolishes altogether, the spontaneous movements.

On the inactivation of adrenaline by mammalian liver *in vitro*. By W. A. Bain and Sylvia Dickinson. (*From the Pharmacological Laboratory, University of Leeds*)

A method has already been described for following the course of inactivation of adrenaline *in vitro* [Bain *et al.* 1937]. In the experiments here reported the inactivating power of different samples of liver, and of samples from different species, is compared. The technique used was the same as in the previous experiments except that the amount of liver used was less (1 g. per 5 ml. instead of 1 g. per ml.). The experiments were carried out at body temperature: the medium was defibrinated cat's blood; and the initial concentration of adrenaline was 40γ per ml. The results were recorded by plotting the amount of adrenaline left against the interval of time between its addition to the blood and liver and the taking of the sample. On an average, seven points were determined in each experiment. The results of forty-two experiments are summarized in Table I.

TABLE I. Approximate mean times in minutes for 50, 75 and 90 p.c. inactivation of adrenaline by livers of different species. Figures in round brackets are the shortest and longest times obtained. Figures in square brackets indicate number of experiments on each species.

P.c. inactivation	Guinea-pig [7]	Man [13]	Rat [7]	Cat [7]	Dog [4]	Mouse [4]
50	12 (8–17)	13 (10–16)	27 (24–31)	34 (21–50)	38 (30–44)	42 (28–48)
75	26 (15–35)	26 (22–31)	53 (49–60)	75 (55–107)	80 (67–100)	79 (53–93)
90	47 (31–61)	43 (35–50)	84 (78–96)	—	—	—

It will be seen that the rate of inactivation effected by the liver is different for different species. There is some variation in the curves obtained from livers of individuals of the same species. But in the guinea-pig, rat, and a set of thirteen samples of human liver, the results lie between fairly well-defined limits about a mean curve, the mean curve being characteristic of the species. It seems possible, therefore, to establish for each of these animals a normal "adrenaline inactivation curve" of liver to which the curve for any given individual will approximate. Wide deviation from the characteristic curve would suggest an abnormality in the adrenaline inactivating power of the tissue.

In three human livers the adrenaline inactivating power was found to be diminished to such an extent, the average times for 50, 75 and 90 p.c. inactivation being 24, 43 and 70 min. respectively. Two of the subjects suffered from arterial hypertension of non-renal origin and the third had a diastolic pressure of 90 mm. Hg, but no other evidence of hypertension. On the other hand, the group of thirteen human subjects includes four cases of chronic nephritic hypertension, in each of which the adrenaline inactivation curve falls within normal limits. It thus seems possible that essential hypertension may be associated with a diminished adrenaline inactivating power of the tissues.

If adrenaline is the transmitter of adrenergic vasoconstrictor nerve activity the raised arterial tension in essential hypertension, and perhaps also in malignant hypertension, may be due to a potentiation of the effects of normal vasoconstrictor nerve activity resulting from the delayed inactivation of the transmitter. We hope to deal with this question in detail later.

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A note on the action of atropine in the bird. By JAS. D. P. GRAHAM.
(*From the Institute of Physiology, University of Glasgow*)

It is a well-known fact that atropine does not dilate the pupil of birds, the musculature of the avian iris being of the striated type. In some experiments on ducks it was noted that atropine appeared to have unexpected effects on the vagal inhibition of the heart and the respiration. It was therefore decided to make a systematic examination of the actions of this drug on the bird.

Depression of the glandular secretions. The chorda tympani nerve having been previously isolated it was electrically stimulated; after a considerable latent period a small quantity of clear viscid saliva was secreted. Atropine sulphate 0·5 mg./kg. was then administered subcutaneously and the stimulation repeated. No salivary secretion was obtained.

Action on the intestine. The contractions of isolated pieces of gut in Tyrode were recorded. It was found that avian gut (proximal jejunum) is not normally very active in its movements. Warmed solutions of drugs were added to the nutrient fluid which was kept at a temperature of 41° C.

Acetylcholine in doses of 20γ in a 100 c.c. bath temporarily increases the tonus; the rate and amplitude of the movements are increased also. Atropine sulphate in a dose of 100γ inhibits the tone of the gut and the effect of acetylcholine is now abolished.

Incidentally it was found that adrenaline in a dosage of 20γ gives rise in every case to a marked but tempofary increase in tone, followed by a loss in tone and some diminution in movements.

Action on the eye. The failure of the pupil to dilate was confirmed, there being no visible effect from repeated instillations of a solution of atropine sulphate 1 in 1000 over a period of 48 hr. Adrenaline 1 in 1000 also had no effect.

Action on the heart. The heart was freely exposed by removal of the sternal plate after reflexion of the pectoral muscles. The bird continued to breathe freely with the aid of the intact cervical and abdominal air sacs. Records were obtained by attaching clips to the tip of the left ventricle and the base of the right auricle. Drugs were administered by the jugular vein.

Stimulation of the vagus produced slowing of the heart; at first the amplitude of movement was increased but later was diminished to complete inhibition. After administration of atropine sulphate (0·5 mg./kg. intravenously) the rate and amplitude of the cardiac movements

were increased. Stimulation of the vagus then resulted in diminution of the amplitude but not of the rate of contraction.

Action on the blood pressure. The blood pressure in the carotid artery was recorded. In a drake a fall in blood pressure from 105 to 70 mm. Hg on vagal stimulation was obtained (Fig. 1A). Administration of atropine sulphate (0·5 mg./kg.) to this bird caused a slight rise of blood pressure followed by a steady fall to 88 mm. Hg, where the pressure tended to remain (Fig. 1B). On subsequent stimulation of the vagus very slight and irregular changes in blood pressure were produced.

Fig. 1. A, B, C. Drake 2 kg. A. Effect of vagal stimulation on respiration and blood pressure. B. Effect of 1 mg. atropine sulphate given intravenously. C. Effect of stimulation of the vagus after atropinization. Interval between A and B 3 min., between B. and C 6 min. D. Drake, proximal jejunum in Tyrode at 41° C. Effect of adding 20γ of adrenaline to the bath of 100 c.c.

The influence of atropine on the blood pressure of the bird agrees with that of the mammal. Apparently the slight residual effect of vagal stimulation of the heart after atropine is insufficient to exert any effect on the blood pressure.

Action on the respiration. The movements of the chest wall in respiration were recorded by the excursions of a stethographic lever.

Atropine sulphate 0·5 mg./kg. of duck administered intravenously caused an increase in inspiratory tone but failed to abolish the apnœa which accompanies stimulation of the vagus (Fig. 1A and C).

Conclusions. It is apparent from these results that the effect of atropine on the bird is not identical with its effect on the mammal. While the absence of pupillary dilatation is explained by differences in histological structure, the dissimilarity in regard to heart and respiration cannot be explained on this basis; the reaction of the gut to adrenaline is noted with interest.

I have to thank Prof. G. M. Wishart of this Institute for performing the operative procedures entailed in this investigation.

Blood histamine in traumatic shock. By A. D. Macdonald and G. Woolfe. (*From the Department of Pharmacology, University of Manchester*)

While it is agreed [Minard, 1937; Code & Macdonald, 1937] that the general level of blood histamine is not increased in acute traumatic shock, Minard claims that the histamine equivalent of the blood from the traumatized limb exceeds that of the normal limb by an average of 80 p.c.

We have compared the histamine equivalents of the blood from normal and traumatized limbs in a series of cats, usually shortly before the death of the animal, with the following results:

Traumatized Limb Blood histamine γ/c.c. (*T*)	Normal Limb Blood histamine γ/c.c. (*G*)	*T*/*G* p.c.
0·034	0·027	126
0·050	0·029	172
0·071	0·066	108
0·033	0·027	122
0·026	0·020	130
0·064	0·059	108
0·084	0·050	168
0·073	0·048	152
0·034	0·028	121
0·038	0·030	127
0·073	0·069	106
0·044	0·029	151
0·040	0·042	95
0·020	0·016	125

It is clear that the increase in the series is less than that found by Minard and not even constantly present. Such rises as are found may well be due to the ischæmia in the swollen tissues, since Barsoum & Gaddum [1935] have shown that after 10–15 min. of circulatory arrest the venous histamine equivalent was approximately four times that of

arterial blood. There would seem to be little justification for assuming that histamine is liberated in significantly increased quantities from the damaged tissues in acute traumatic shock.

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